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Laboratory of Experimental Surgery

of the University of Liège

Professor F Albert

Dr G Lejeune Ledant

(18th 21st of March 1959)

LES CONGRES ET COLLOQUES DE L'UNIVERSITE DE LIEGE

BIOLOGICAL PROBLEMS OF GRAFTING

A SYMPOSIUM

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FOREWORD

Now that our Colloquium has come to an end and that we are preparing for the very near future the publication of the proceedings, it is a pleasant task to thank once more all those who helped us to make of our Conference a great international success.

We must first of all express our deep gratitude to the C.I.O.M.S. (Council for International Organisations of Medical Sciences) and to the C.A.P.U. (Commission Administrative du Patrimoine Universitaire de Liège) for their important, spontaneous and generous support without which our Colloquium would never have seen daylight.

The C.I.O.M.S. showed once more its particular interest for the organisation by its member associations (in this instance the Société Internationale de Chirurgie) of scientific meetings of international interest principally those requiring the participation of research workers of various disciplines.

For many years now, the C.A.P.U. has placed on its programme the organisation by its teaching staff of the International Colloquia of the University of Liège. This present one was an example among many others.

The combined sponsorship of both these Organisations enabled us to make of this Colloquium a wonderful performance owing to the important participation from multiple countries, the international reputation of all the distinguished members, the high scientific level of the communications and the special interest of the discussions. We do hope that neither will have the slighest regret for having trusted us and we must thank them very sincerely.

The proceedings of our Colloquium will reach a very wide audience not only of those interested in the biological problems but also those who are mainly concerned with the practical applications to humans. All the communications will be published in the Journal of the Société Internationale de Chirurgie and the Chairman and one other member of the Colloquium will report to the next congress of this Society the general conclusions of the communications and discussions and their repercussion on the surgical problems of grafting.

We also owe very much to all those who brought us their invaluable help

First of all Professor Medawar who spared neither help nor advice before during and after the Colloquium, which he presided with all his prestige elegance and cordiality His authority and his unfailing willingness enabled us to fulfil our secret wish of collecting, at the end of the conference, all the corrected discussions We like to tell him again our admiration and gratitude

Professors Hašek (Tchekoslovakia) Klein (Sweden) Knake (Germany), Owen (U.S.A.) Simonsen (Denmark) van Bekkum (Holland), and Voisin (France) were all excellent deputy-chairmen and directed the discussions with competence and skill. To them too we are much indebted.

We must also sincerely thank all the participants who gave us during this Colloquium the very latest results of their splendid researches Thanks to the cordial atmosphere, the intimacy of our meetings and the frank spirit of friendship one felt right throughout all the Colloquium the sessions retained from beginning to end the same high scientific level and the open, spontaneous discussions were of a wonderful standing indeed

We are sincerely thankful to all those who helped to make this Colloquium really what it was and we do hope it will be but a link of an endless chain.

We would not like to close this list without expressing our gratitude to the secretaries for the tremendous task they performed having all the discussions typed ready to be corrected immediately by the authors all this while the Conference was going on their merit was the greater as all the work they did was « off stage » In name of all the members we like to express them our admiration and warmest thanks

The goodwill and enthusiasm of every one were the means by which the proceedings may be published in an exceptionally short time

And finally we are pleased to add our personal acknowledgment to all the members of our Laboratory of experimental Surgery They all contributed a great and prolonged effort towards the most perfect possible organisation of the Colloquium.

F A

Séance d'ouverture du Colloque sous la présidence du Recteur de l'Université

Introduction Professeur F. ALBERT

Monsieur le Recteur,

Laissez-moi vous dire, au nom de tous les participants, combien nous sommes heureux et fiers de vous voir présider la séance d'ouverture de ce Colloque consacré à l'étude des Problèmes Biologiques des Greffes.

Nous savons combien votre temps est précieux, nous connaissons vos importantes et absorbantes préoccupations rectorales; nous attachons d'autant plus de prix à votre geste. Mais, nous n'oublions pas, aujourd'hui moins que jamais, que vous êtes aussi le Biologiste de notre Université. Votre présence nous prouve une fois de plus, si c'était nécessaire encore, combien vous aimez encourager toutes les initiatives, même modestes, capables pourtant de jeter un rayon de lumière sur cette Université dont vous dirigez la destinée avec tant d'éclat.

Au nom de tous les Collègues étrangers et belges qui ont bien voulu nous apporter leur précieuse collaboration, je vous prie d'agréer, Monsieur le Recteur, l'expression de notre très vive gratitude.

Je voudrais aussi adresser un cordial merci à notre Doyen de la Faculté de Médecine de Liège, qui se dévoue sans compter, avec le plus grand succès d'ailleurs, à la prospérité et à la grandeur de sa Faculté.

Et maintenant, Monsieur le Recteur, quoique notre Université soit d'expression française, je voudrais, par délicatesse vis-à-vis de la majorité de nos Collègues étrangers, vous demander l'autorisation de continuer ou du moins d'essayer de continuer en anglais.

* * *

It is a very great pleasure for me to welcome all the distinguished Colleagues from abroad who have shown so much enthusiasm in answering our invitation. Dr Snell is very sorry, but had to give up his first intention of coming to Liege, because he had too many obligations this year; our Swiss Colleague Allgöwer has asked to be excused as he is just leaving for a journey to the United States. Professor Gaillard of Holland was recently named Dean of his Faculty and regrets not to be able to attend our conference.

All the other Colleagues we invited are assembled here many of them after a very long journey I thank you all for having accepted our invitation.

We are honoured to have among us two delegates of the Transplantation Committee of the National Academy of Sciences — National Research Council of Washington. May they be assured of our gratefulness and express it to their Academy

My best and very sincere thanks go to Professor Masin President and representative of the Executive Committee of the C.I O M.S (Council for International Organisations of Medical Sciences) who has accepted to lay aside for a short time his innumerable international obligations to attend at least a part of our Meeting

I also thank my old friend Professor Martin who came from Brussels to represent the « Société Internationale de Chirurgie » at this opening session.

* * *

You all know my dear Colleagues, the importance of the problem of grafting for the surgeons and everybody can imagine the enormous progress it would mean to medicine and to surgery to succeed in grafting *living tissues and organs.*

Host intolerance has compelled the surgeon to keep to autografts each time it was possible to find on the patient himself the materials needed for substitution

For bone grafts, this has never presented great difficulty because the skeleton easily supplies all sizes and forms of grafts. For vascular grafts the problem was far more difficult to resolve, and the surgeon had to take elsewhere the bloodvessels the host himself cannot do without. It was then necessary to try and make this graft tolerable for the host by various treatments that were meant on one hand to ensure their conservation on the other especially to reduce their antigenicity responsible of intolerance reactions.

And for a while, the surgeons misled by the histological aspects of the grafts thought that this grafts, *although losing their antigenic properties* were still alive. They realised in the end that in fact this homografts or even heterografts accepted by the host, were really dead grafts that is to say prostheses on which the host himself would rebuild and remodel tissues that had to be replaced.

This rather disappointing statement was going to decide experimentators and surgeons to try plastic prostheses instead of vascular homo- or heterografts.

And finally except for certain grafts that succeeded in privileged regions, but cannot be transferred to humans and for a few successfull

tiny grafts or cultures of hormonal tissue, host intolerance remained an insuperable obstacle as far as living grafts were concerned

As we know tolerance may be induced in several ways but none of these is sufficiently devoid of severe risks to allow immediate human applications.

We are all aware of the supreme difficulty of the biological problems we are facing although being among the most fascinating of our time. Every organism has its own means of self defence struggling for life against all agressions coming from outside or from inside. Since the pasteurian era bacteriologists and immunologists joined all their efforts in order to strengthen or to produce artificially these reactions against all infectious agents. By helping the organism in the normal way of its spontaneous defence, vaccination and serotherapy gave spectacular results. But slowly other reactions were observed this time much less favourable to the patient.

All the multiple and various allergic reactions added themselves to the accidents of anaphyllaxy all of them causing more or less severe perturbations sometimes fatal for the patient.

In our own problems of grafting a new element that of histo-compatibility was discovered. We are obliged all the way long to take in account the hereditary factors responsible for host reactions towards homografts and acting against or in favour of tolerance.

For the last few years another fact stood out these same heredi tary factors may act in an inverted way A host spontaneously or arti ficially tolerant to homografts may become victim of a new disease, the « runt disease » and « homologous disease » due to the reactions of the grafted cells against their host if the latter has some antigens absent in the donor

At each apparent progress at each further step new difficulties sprang up. There is nothing surprising to that as to create tolerance to homografts we must be able to inhibit the normal host reactions which have been so successfully exploited against infectious diseases.

Do not we see agammaglobulinemic patients tolerate their homo-grafts and undergo for the same reasons without means of defence, the assaults of the pathogen agents ?

No doubt, one can create tolerance by injecting homologous cells of the future donor to the recipients if these are still immunologically immature or to others, the immunological reactions of which have been paralysed by lethal irradiation.

So far this tolerance only seems possible thanks to the survival of living cells used to prepare the host. That cellular chimaerism in the host seems indispensible. But it is just in this case that one must fear homologous disease with its fatal consequences some of you tend to

avoid this disease by injecting homologous foetal or embryonic immature cells.

Creation of tolerance should be possible by means of cells incapable of reacting against host antigens. Perhaps, shall we one day find *the possibility of inhibiting the intolerance reactions* by means of non-living and non toxic substances, acting in a specific or non specific way and perhaps preferably non specific ?

Many of these problems remain still unsolved. We have met here at this Colloquium to confront the results of our researches, to discuss them freely in order to help one another and try to find new suggestions for our future research. And we may also hope that the results acquired *during this conference will* be of some utility to those who are highly interested in the practical applications of grafting but do not have the opportunity of cooperating towards the solution of the biological problem, either through lack of time or lack of appropriate means for experimental work.

The « Société Internationale de Chirurgie » had put on the programme of its meeting that will be held in Münich next September *the problem of transplantations and grafting. Surgeons want to* be informed of the actual situation of the biological problems of grafting. We must show the surgeons how numerous and complicated are the problems, how difficult their solution and how cautious one ought to be before trying to apply to humans certain successful results obtained in our experimental work.

During one of the meetings, we suggested to the Executive Committee of the C.I.O.M.S. the idea of a Colloquium on these problems, to be held not during the Congress as they had always done but several months before, in order to enable us to bring to the Congress the benefit of our discussions. Our suggestion was greeted with much enthusiasm and sympathy by the President and all the members who immediately accepted to sponsor this conference and help us for the organisation.

The « Commission Administrative du Patrimoine de l'Université » aware of the importance of this meeting agreed to put it on the programme of the Colloquia of the University that we owe to the constructive energy and the unfailing initiatives of our dynamic Rector. We wish to thank warmly Professor *Maisin* who *represents here* the C.I.O.M.S. and the Rector President of the C.A.P.U. for the precious help and the generous support they have given us in realizing our plans. We would like to ask them both to express to the Institution which they represent our deep and sincere gratitude.

Thanks to their moral and financial help, we were going to be able to overcome all the difficulties organizing this Colloquium to the invi

tation of which you have answered with such enthusiastic unanimity and spontaneity that our efforts have been fully rewarded. Thanks to all of you I am sure we shall not disappoint those who have accepted to give us their help and trust.

And now my dear Colleagues the best we can do is to start work ing without further delay We had at first decided to spend four whole days on this Colloquium. I think, from the number and the importance of the papers that we will quite need all of our time. We have given you a lot of work, and you must excuse us for having asked you so soon the copies of your papers

But if our Colloquium must fill the purpose given to us by the C.I O.M.S., I must be able to have all the papers printed soon enough to be given out in time to the members of the « Société Internationale de Chirurgie » But there was, I have told you another reason I wanted the interest of the Colloquium to be in the discussions as well as in the papers themselves Whe should especially like these discussions to be as lively and as open as possible, and everyone to take part without the slightest restriction.

As you see, we are going to try recording all the discussions and the secretaries will do their utmost to give all those who have taken part in the discussions, the text of the recordings. Everyone would bring to it all the corrections he wishes to Some may eventually ask that a refle xion of theirs should not be recorded.

As far as it is possible, we should like each speaker to agree with the final texts before we separate. This will enable us to print them within the shortest time, while the Conference is still fresh in mind and not serve it after several months as a warmed up dish perhaps already out of fashion.

Of course, our Colloquium will be held as a real round table Con ference and the order of the papers has been established only so as to group them and thus make discussions easier We should like everyone to feel equal to the other members. This will take away some of the reserve that the younger participants may feel compelled to show when they take part in the debates

A round table conference does not absolutely need a president but we must have someone to lead and to animate the discussions and for many reasons I am proposing to you that Professor Medawar with the assistance of a deputy-Chairman for each session should be entrusted with this task. He has brought such a precious help in the organisation of this Colloquium and if we are assembled here in Belgium I would like him not to feel too far from home as he is already a member of our Royal Academy of Medicine and to feel quite at home at the University of Liège of which he was elected recently a « Doctor Honoris Causa »

Iso-antigens

P B Medawar

University College University of London

Introduction.

The problems that will be touched upon in this article can be put in the form of questions What is the chemical nature and the exact intracellular and histological distribution of the antigens that cause transplantation immunity ? Are the antigens that cause transplantation immunity the same as those which are responsible for the formation of humoral iso-antibodies ? If not do they differ in respect of their determinant groups or in the nature of their subsidiary attachments ? Can the injection of pure red cells incite transplantation immunity ? In what form does the antigenic stimulus reach the centres of immunological response ? How soon does transplantation immunity take effect and how long does it last ?

Very few of these questions can yet be answered. Yet it is not surprising that this should be so The antigens responsible for transplantation immunity may be as complex as the bacterial endotoxins and are very unstable they must be extracted from the tissues of inbred animals which in most laboratories are in chronically short supply and even the simplest method of identifying them involves a lengthy and laborious biological test which in one important respect (see below) is very insensitive. There is, indeed only one aspect of research on iso-antigens which we can contemplate with any sense of satisfaction the genetical analysis begun by C.C. Little and developed with conspicuous success by P A Gorer and G.D Snell and their respective colleagues. The Mendelian analysis of antigenic differences does not of course, give us any information about the chemical nature of the antigens themselves but it has brought to light three facts that bear directly upon the answers to the questions put above The first is that at least

some of the antigens responsible for transplantation immunity have a common genetic determination with antigens that elicit the formation of humoral antibodies. Unless we suppose that one allele can imprint two different immunological specificities upon its cellular products we must infer that the antigens responsible for transplantation immunity on the one hand and for the formation of humoral antibodies on the other hand differ—if they differ at all—only in respect of subsidiary groupings that favour the one pathway of response rather than the other The second is that the antigens are very numerous (for skin homografts see Barnes and Krohn [3]) and the third that they are of very unequal strength

What follows is a summary almost in note form, of the kind of evidence that may help us to arrive at the answers to our questions

IDENTIFICATION OF ISO-ANTIGENS

1 *Antigens forming humoral antibodies* (« *H-antigens* ») H antigens can be identified by their power to provoke the formation of humoral antibodies or to absorb them from immune sera. The humoral antibodies themselves belong to several different species (see Gorer [23] Amos and Day [2] Feldman and Sachs [10] Hildemann and Medawar [28]) they have been identified as haemagglutinins (Gorer [21 22]) haemolysins (Hildemann [27]) leukocyte agglutinins (Amos [1]) cytotoxins (Gorer and O Gorman [24]) and as protective antibodies (see Gorer [23]). In addition, humoral iso-antibodies which may or may not be haemagglutinins can be identified by their power to secure « enhancement » (Kaliss [30] [31]) and this biological property it should be noted underlies the only sustained attempt that has yet been made to discover the chemical nature of H antigens (Kandutsch and Reinert Wenck [32])

2 *Antigens causing transplantation immunity* (« *T antigens* ») The main hindrance to the analysis of T antigens is the fact that only « biological » (as opposed to serological) methods can be used for their identification These biological methods are (i) their ability to produce transplantation tolerance (Billingham Brent and Medawar [8]) a method which is too lengthy and quantitatively too insensitive for systematic use—particularly in the analysis of cell free antigens which unlike living foreign cells, cannot provide the chronic antigenic stimulus that seems to be required (ii) their ability to provoke transplantation immunity i e. to affect an animal in such a way that it gives a « second set » response when challenged with living homografts from the antigen donor and (iii) their ability to produce the cutaneous hypersensitivity reaction described by Brent Brown and Medawar ([12])

Iso-antigens

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and herein. It has not yet been formally proved that the antigen which elicits this third reaction is identical with the antigen that causes transplantation immunity Cutaneous hypersensitivity reactions are, unfortunately at the mercy of agents that cause non specific inflammation, and for this reason the method will not be easy to adapt to a chemical identification of the T antigens The whole of our present knowledge of the chemical nature of T-antigens is in fact based upon the application of the second method i.e. upon their power to provoke transplantation immunity (Billingham Brent and Medawar[9, 12])

Histological distribution

The transplantation of living tissues produces both humoral immunity and transplantation immunity H antigens are widely represented in the fixed tissues and some, at least, are on the surfaces of red cells (review by Gorer[23]) My colleagues and I have failed to produce skin transplantation immunity or tolerance with purified red cells whether intact or disintegrated, and believe that the antigenicity of whole blood is fully accounted for by its content of leukocytes. Using a very different test-system and tumour homografts rather than skin Barrett[4] has nevertheless confirmed his earlier observations on the presence of a T-antigen in or on red cells Red cells apart, there is no disagreement about the widespread distribution of T-antigens in living tissues. Inasmuch as pure blood monocytes can cause transplantation tolerance in chickens (Terasaki unpublished) and pure epidermal cells can elicit transplantation immunity in rabbits (Billingham and Sparrow[14]) there is no need to suppose that lymphoid cells are the prime movers in transplantantion immunity (cp Snell[44]) Experiments based upon the principle of acquired tolerance do however make it very unlikely that any tissue contains T antigens that are not fully represented in lymphoid cells the neonatal injection of splenic cells, for example, is known to produce tolerance of homografts of skin (Billingham and Brent[8]), thyroid (Woodruff and Sparrow[46]) ovary (Krohn[33]) and adrenal cortex (Medawar and Russell[36]) (Somewhat weaker evidence that *all* nucleated tissues have a qualitatively similar representation of T antigens has been reviewed by Medawar and Russell[36]) Living lymphoid cells are adept at producing both humoral immunity and transplantation immunity partly perhaps because their « homing instinct » (Mitchison[39]) ensures their reaching the reaction centres of their host This cannot be the whole explanation for lymphoid cells are particularly good sources of cell free enhancing antigens (see Snell[44]) and T antigens (see below) Cell for cell skin homografts are just as effective as living lymphoid cells in producing transplantation immunity

ROUTE OF ADMINISTRATION AND DOSAGE OF ANTIGEN; TIME OF ONSET AND DURATION OF IMMUNITY

These four variables are partially interdependent: it is therefore impossible to make a significant statement about one without specifying the values attached to the other three.

1. *T-antigens.* The effectiveness of the different routes of immunization must depend upon many factors: the degree to which they give access to the centres of response and the number of centres that are activated; the mobility of cells if living cells are used; the presence or absence of local changes (e.g. granuloma formation) which may influence the nature or tempo of the immune response; and so on. Disregarding matters of parochial interest—e.g. the long survival of homografts lying wholly within the meninges, a dispensation presumably due to the absence of lymphatic drainage—the most important distinction is between the intradermal and subcutaneous routes on the one hand and the intravenous and intraperitoneal on the other. In guinea pigs and mice lymphoid cells seem to be about equally effective through all these routes, the degree of effectiveness being judged by the intensity of the second-set response to skin or tumour homografts transplanted eight to ten days after the sensitizing injection (Billingham, Brent and Mitchison [13]). There is an unexplained mystery about the intravenous route in rabbits, for the intravenous injection of whole blood or of dissociated epidermal cells so far from curtailing the life of a second-set skin homograft may actually prolong it (Billingham and Sparrow [14]; cp. Medawar [15]).

The way in which the strength and duration of transplantation immunity varies with antigenic dosage has been studied anew by Billingham, Brent, and Medawar (unpublished). As earlier work on skin homografts in rabbits had led us to expect, the dosage/response curve is distressingly flat: a study of the effects produced by the intraperitoneal injection of foreign homologous splenic cells in mice shows that there is little to choose between the strength and duration of the immunity produced by 5m and by 0.25m cells—a twentyfold difference of dosage. (This insensitivity to dosage is a severe hindrance when using the magnitude of the second-set response as a method of assay for cell-free antigens.) With CBA mice as recipients 0,125m A line splenic cells is about the smallest number which, injected intraperitoneally, will cause 50 % of skin homografts transplanted three days later to break down completely within six days. At the other extreme, there is at present no evidence that T antigens in any form can be administered in immunologically paralyzing doses. Castermans [17] finds that very

high doses of foreign homologous splenic cells produce a wasting syndrome in mice that is probably akin to « runt disease » (Billingham and Brent[5, 6]) and there is some evidence that « parabiotic disease » belongs to the same etiological family (see Finerty[26] Nakic and Silobrcic[43])

The time of onset of transplantation immunity in « virgin » animals has been studied by several methods among them the following (a) by direct observation of scale homografts in goldfish (Hildemann[34]) (b) by finding out how long a first-stage homograft must be present before it can affect the behaviour of a second-stage homograft (Converse, Ballantyne and Woisky[18]) (c) by examining the competence of regional lymph nodes to secure « adoptive » immunization (Mitchison[39]) (d) by the hypersensitivity test described herein by Brent Brown and Medawar and (e) by using antibody formation as a criterion of cellular viability and finding out at what stage a passive host can distinguish between homologous and isologous lymphoid cells (J.K. Dineen and B T Perry unpublished cp also Mitchison and Dube[41] Harris and Harris[35]) The last of these methods has not yet been fully adapted to answering the particular question we have in mind the most sensitive method (a) shows that under the appropriate conditions goldfish can discriminate visibly between autografts and homografts within 48h of their transplantation. The other methods suggest that transplantation immunity begins to exercise crudely discernible effects about three days after antigenic stimulation Obviously the cellular changes that anticipate them must happen earlier still

Very perplexing problems are raised by an analysis of the *duration* of transplantation immunity The sensitivity produced in mice by the transplantation of a skin homograft about 1 cm square is still very obviously in force 240 days later (Billingham, Brent and Medawar[7] and unpublished work). The transplantation of a very thin skin homograft only 1½ mm square (containing perhaps less than 50,000 viable epidermal cells) will cause the majority of second-stage homografts transplanted 30 days later to break down completely within six days—the maximal response that can be recorded in our test system It is far otherwise with the sensitivity produced by intraperitoneal injections of 0 25m to 5 om splenic cells or of cell free splenic antigen Here the peak of sensitivity is revealed by skin homografts transplanted as soon as three to five days after the sensitizing injection. If the test is deferred until the 20th day sensitivity can be seen to have declined to a small fraction of its peak value. What is the cause of the profound difference between the duration of sensitivity produced by skin homografts on the one hand and living splenic cells or splenic extracts on

the other? One possibility is that living splenic cells eventually produce « enhancing » antibodies which interfere with the expression of sensitivity in a purely biological test of this kind (cp Kaliss[21]). This explanation is probably inadequate, for cell free antigens produce no detectable humoral antibodies at dosages much higher than those which were used in these experiments (Hildemann and Medawar[20]). A second possible cause of the difference is that skin homografts like other solid homografts concentrate their antigenic action upon a small group of regional nodes (Mitchison[25,26], Billingham, Brent, and Medawar[7]) whereas splenic cells introduced by the intraperitoneal route may exercise their effects more diffusely. This too cannot be the whole story for the sensitivity produced by an intradermal injection of cell free splenic antigen decays just as rapidly as that which follows an injection by the intraperitoneal route. A third possibility is that a vascularized « solid » homograft becomes the seat of granulomatous changes which somehow prolong the immunological response. Whatever may be the answer we can no longer assume that transplantation immunity is of necessity long lasting.

Mitchison[26] found that the competence of regional nodes to transfer the sensitivity produced by a regional tumour homograft in mice began 3 to 5 days and ended 10 to 15 days after its implantation. These results cannot be compared directly with our own evidence of the rise and fall of sensitivity in mice which had been actively immunized by splenic cells. In Mitchison's experiments the active immunity produced in the mice which were the donors of the transferred cells long outlived the competence of the regional nodes to transfer the immunity to other mice. Presumably then, loss of competence of the regional nodes was accompanied by a slow activation of other lymphoid centres, i.e. by a widespread diffusion of what began as an almost exclusively regional effect. We need not assume that activated lymphoid cells are permanent residents in any one lymph node; they may well migrate round the body and find temporary homes in other lymphoid centres.

Evidence that anything akin to a true secondary response occurs in transplantation immunity is very meagre. It is a pre-existing and not a re-awakened sensitivity that causes « second set » homografts to break down so quickly; they *may* provoke a secondary response—in respect of humoral immunity they are known to do so—but their own short lives are not a consequence of having done so. Mitchison[26] alone has obtained clear evidence of a secondary response on transplantation immunity. Though it arose quickly it was rather feeble; this is not to be wondered at because prompt breakdown of the vasculature of second-stage grafts must hinder the access of antigenic matter to the regional nodes.

2. *H-antigens.* The immunological response to antigens which elicit the formation of humoral antibodies seems to be entirely orthodox in tempo and duration and in respect of the distinction between primary and secondary responses the available information has been discussed at length by Gorer[23] and Snell[44] and need not be recapitulated here. Titres of serum antibodies are, of course, incommensurable with homograft survival times, but there is nothing to prevent us comparing the kinetics of humoral immunity and transplantation immunity Mitchison and Dube[41] showed that the ability of activated lymphoid cells to transfer heightened resistance to a tumour homograft arises earlier than their ability to transfer agglutinin production and declines more quickly Gorer[23] denies that these results are generally valid he finds that humoral antibodies in mice may often be detected as early as the third day and before it is possible to detect a homograft reaction His complaint that Mitchison and Dube may have used an insensitive method of detecting haemagglutinins can hardly affect their main contention that the *peaks* of transplantation immunity and humoral antibody formation are reached at different times Perhaps the matter should be re investigated on mice actively immunized by methods which are known to produce a transplantation immunity of rapid onset and quick decay e.g by the intraperitoneal injection of a million A line splenic cells into CBA mice.

It is now well known that tissues killed by drying in the frozen state can provoke the formation of haemagglutinins, though less effectively than living cells it is indeed to this or to a closely related property that they owe their power to secure « enhancement » (Kaliss[29]) There is a good deal of evidence, reviewed by Gorer[23] and Snell[44] that the *quality* and not merely the degree of the immune response may vary with the dosage of dead tissue. The results vary from one pair of strains to another and from one tumour to another but the dosage effect when it exists, is usually such that low doses raise resistance to homografts and high doses lower it. It has been reported that as little as 0.5 micrograms of dried tissue can provoke resistance (Kaliss[29]) The test system used to reveal such an effect must be of extraordinary sensitivity for even with a tissue as rich in cells as the spleen 0.5 micrograms can hardly represent the dried residue of more than 5,000 cells. The problem of the mechanism of the dosage differential is still further complicated by the fact that qualitatively different responses may be associated with different titres of serum antibody thus Kaliss and Gorer (see Kaliss[31]) have shown that the degree of enhancement produced by the passive transfer of antisera may sometimes vary *inversely* with serum dosage, and that high doses so far

from enhancing, may sometimes inhibit the growth of tumour homografts. Considered as a whole, there are at present too many variables in these experiments for us to form any clear picture of the mechanisms at work.

TIME OF ORIGIN IN DEVELOPMENT

(i) *T-antigens* The injection of « embryo mince » was much favoured by early students of transplantation as a method of conferring protection against homografts of tumours. The fact that it does so cannot be taken to imply that T antigens are manufactured before birth, for the embryonic tissues might have undergone further development *in vivo* before they took effect. This objection applies to all methods of timing the origin of T antigens that make use of the second-set response. It was partly but not completely met by Billingham Brent and Medawar's [4] demonstration that the injection of 11½-day mouse embryonic tissue into adults of a different strain causes the breakdown, within six days of skin homografts transplanted three days later The test extended over a total of nine days and was thus complete before, had they been spared, the 11½-day embryos would have been born The only formally satisfactory method of testing the antigenicity of embryonic tissue is to investigate their power to induce tolerance, the test being arranged in such a way that embryonic cells are injected very near the end of the « adaptive period » of their hosts (The purpose of doing so is to give the embryonic tissue the very minimum of time to grow up *in vivo*) By these methods it has been shown that T-antigens are present in 6-day chicken embryos (Terasaki and Medawar unpublished) and 11-day mouse embryos (Billingham and Silvers, unpublished) Very recently Toolan [45] has reported that homografts of the skin of very young rabbit embryos often survive much longer than homografts of adult skin. It seems hardly possible that this should be due merely to the absence of T-antigens for even if they were absent to begin with they should have made their appearance when the embryonic skin underwent further differentiation on its host. Further analysis along orthodox lines will doubtless show whether the embryonic skin owes its privilege to a failure to excite an immune response or to some kind of protection from its consequences. It is tempting and by no means without precedent, to attribute the anomalous behaviour of embryonic skin to the occurrence of a phenotypic transformation—e.g to a competitive replacement of the antigens present on the cellular surface. The fact that this does not occur when embryonic cells are used to procure tolerance (see Billingham Brent, and Medawar [4]) is hardly relevant for in this context selective pressures need not be at work

oxyribonuclease when in this form. We accordingly worked for some time on the assumption that the antigens were probably DNA proteins or other substances (e.g. lipoproteins) integrally associated with the chromosomal complex. The idea that antigenicity depends upon the participation of DNA can now be discounted on internal evidence, and the important demonstration by Kandutsch and Reinert Wenck [32] that purified DNA-proteins still contain hexosamine—and so by inference mucoid substances—makes sense of the occurrence of T antigens in nuclear matter. We cannot now say with conviction that T antigens are wholly or even predominantly nuclear in origin: the polysaccharide containing moiety of our nuclear preparations may be purely adventitious. It is true that, in our hands, cytoplasmic preparations are still obstinately non-antigenic: unfortunately there is no manipulative technique which can be relied upon to make cell membranes appear in any one well-defined physical fraction of disintegrated cells.

Although we have not yet secured *tolerance* by the neo-natal injection of cell free antigenic matter we still do not believe that our failure can be blamed upon anything except the difficulty of maintaining a sufficiently powerful antigenic stimulus for a sufficient length of time.

2. *H-antigens*. We start here with the firm knowledge—for which we are mainly indebted to W. T. J. Morgan and E. A. Kabat—that all the human blood-group substances which have been chemically identified are amino-acid polysaccharide complexes and that antigens of this kind are ubiquitous in distribution. The rapid progress of their work depended partly upon the existence of a simple and reliable method of assay (based on haemagglutination inhibition) and partly upon the abundance of antigenic matter provided by hog gastric mucin and human ovarian cyst fluid. Not even the facilities of Bar Harbor could ensure so copious a supply from inbred mice. Nevertheless simple indirect methods (cp. Morgan and Watkins [42]) should be able to decide whether or not the red-cell receptors corresponding to the H-2 antigens of mice are mucoid substances and it is surprising that no one has yet put them to this use. As matters stand our knowledge of the chemical nature of H antigens is confined to those which secure enhancement. It has been just as difficult to acquire as information about T antigens and for the same reasons: the instability and complexity of the substances involved and the use of biological methods to detect them. (Indeed it may even be more difficult for a biological test based upon enhancement must be more easily prey to non-specific influences.) The cogent study by Kandutsch and Reinert Wenck [32] showed that enhancing activity is present in various cytoplasmic fractions in nuclear fractions and in fairly pure DNA protein. The difference of potency be

tween the most active and the least active fractions was only on the borderline of statistical significance. Hexosamine was present in all fractions Activity was destroyed by the periodate ion and by the action of certain protein denaturants the antigens present in insoluble fractions resisted the action of ethanol and ethanol-ether at 50-60 C, and enhancing activity was not destroyed by drying in the frozen state. Kandutsch and Reinert Wenck infer that the enhancing antigens are probably carbohydrate protein complexes a conclusion that fits well with the indirect evidence cited above.

It seems likely that living cells particularly lymphoid cells are better than dead cells or cellular extracts in producing humoral anti bodies. Using mice in the combination A to-CBA Hildemann and Medawar[24] produced haemagglutinin and haemolysin titres of 1024 by five suitably-spaced injections of only 0.56m splenic cells, the number expressed from about 0,25 mg wet weight of splenic tissue. Much larger doses of lyophilized tissue were much less effective. There is no doubt at all that cell free extracts are much less potent T-antigens than living lymphoid cells or solid homografts The comparison between dead and living preparations is however inexact. The antigenicity of any pre paration depends (a) upon its antigenic content and (b) upon the proportion of it which can reach a centre of immunological response. The homing instinct of living lymphoid cells, and the fact that the lym phatic connections of solid homografts open a direct pathway to the regional nodes go a long way towards explaining the superiority of living cells.

Discussion

The information summarized above (admittedly only a fraction of the knowledge at our disposal) does not yet fall into a coherent pattern. There is unfortunately a long-standing conflict of interests between those who are interested in transplantation immunity as such or for its possible surgical import and those who are specially concerned with the immunological reactivity of tumours and it just so happens that it roughly corresponds with the division of interest between the cellular and the humoral components of the immunological response. Not much progress will be made if we continue to study either one of them to the exclusion of the other For example, it is most important to find out whether or not splenic cell extracts known to contain T-antigens (see above) can if suitably administered produce humoral anti bodies and enhancement and whether or not they can absorb humoral antibodies from sera in which they have been caused to appear by efficacious means Hildemann and Medawar[24] have made a beginning

with this problem splenic nuclear fragments though adept at producing transplantation immunity produced no detectable haemagglutinins or haemolysins after repeated injection at suitably-spaced intervals into mice, and neither they nor a « solubilized » antigen made from them absorbed humoral antibodies from the sera of mice which had been immunized by living splenic cells. (We hope to repeat this work with the less crude antigenic preparations that are now available) Now that Kaliss has proved that it is mediated through the action of a humoral antibody it is equally important to come to some firm conclusion about the mechanism of enhancement Theories of enhancement are of three kinds (see Billingham Brent, and Medawar [10] Kaliss [21]) humoral antibodies may affect the inception of the immune response or the peripheral expression of the immune response or (as in acquired tolerance) the immune response itself. We and Snell incline towards accepting the first of these possibilities but Kaliss believes that the second is nearer the truth. My colleagues and I are now studying the effect of hyperimmune sera on soluble T antigens, in the hope that this will throw light on the mechanism of enhancement (see Appendix)

One antigen or many? The balance of evidence (no one part of it decisive) turns in favour of the view that T antigens and H antigens are different substances with the same genetic and immunological specificity and therefore with the same determinant groups The immunologically active components are probably amino-acid polysaccharide complexes—« mucoids » in Morgan's terminology The *kind* of response they elicit, whether cellular or humoral could well depend upon subsidiary molecular attachments. Snell [44] whose discussion of the problem is particularly cogent suggests (following Kandutsch) that lipid attachments might favour the cellular rather than the humoral pathway of response (*) I myself find it difficult to understand why the power of an antigen to secure enhancement should depend on the protein attachments envisaged by Kandutsch and Reinert Wenck Morgan has shown that in certain systems purified mucoids are admirable antigens they are substances of comparatively low molecular weight (about 250,000) and are not inactivated by substances that denature proteins. Now that new methods have been devised for the extraction of immunologically active mucoids from ordinary tissues (Lawton McLoughlin and Morgan [34]) it should not be difficult to find out if they can excite the formation of humoral antibodies. They should at least be able to block the action of humoral antisera at best they might interfere drastically with the cellular component of the immune response.

(*) To the best of my knowledge attempts to excite transplantation immunity by combining lyophilized tissues or red cells with mycobacterial adjuvants have been unsuccessful (unpublished work of Mitchison and of R. D Owen and Brent)

One pathway of response or many? If iso-antigens are of at least two kinds do their actions converge upon one system of immunologically reactive cells or do they employ different pathways of response from the outset? By analogy with certain other immunological responses, we might construe the cellular mode of response as being no more than an episode in a process that eventually leads to the formation of serum antibodies but there is little internal evidence in favour of this interpretation and much against it. The humoral antibodies themselves are of more than one kind are these, too the products of different clones of antibody-forming cells? In a situation like this, where we are obliged to dissect a plural immune response, there is only one singly decisive way of *distinguishing between two immunological pathways* by using the principle of acquired tolerance to block one but not the other Here, too only a bare beginning has been made Billingham Brent and Medawar [11] showed that the injection of newborn chicks with adult homologous red cells could depress the formation of iso-agglutinins in response to active immunization in later life without having any discernible effect upon the strength of transplantation immunity

In this discussion the space that should have been occupied in answering the questions set at the beginning of the paper has in fact been occupied by asking more. Perhaps no other comment is needed on the present state of our knowledge of the iso-antigens.

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APPENDIX.

Reference is made on p. 82 to experiments on the effects of hyper-immune sera on the activity of cell-free antigens. The following is a summary of their results so far as they have gone.

CBA mice were hyperimmunized by six weekly injections of the cells expressed from 12½ to 100 mg A line lymphoid tissues (approximately 25 to 200 million cells). Six or seven days after the last injection we studied:

(a) The immune status of the hyperimmune CBA mice, as revealed by the intensity of their reaction upon A-line skin homografts.

(b) The effect of the transfer of hyperimmune serum to normal CBA mice a few days before grafting with A line skin.

(c) The power of the hyperimmune serum to counteract the effects of A line soluble antigen when both were injected into normal CBA mice a few days before the transplantation of A line skin.

(d) The power of lymphoid *cells* from hyperimmune CBA mice to « absorb » or otherwise inactivate A line soluble antigen as tested by its power to excite transplantation immunity in normal CBA mice.

(e) The capacity of lymphoid *cells* from hyperimmune CBA mice to transfer immunity « adoptively » to normal CBA mice—again measured by the power of the mice so treated to react upon A line skin homografts.

(f) The effect of hyperimmune serum upon the power of hyper immune cells to transfer immunity adoptively to normal CBA mice according to schedule (e)

The following results have been obtained

(a) CBA mice which have received repeated weekly injections of 25 200m A line lymphoid cells react much *less* strongly upon A-l skin homografts than CBA mice which have received a single injecti of so few as 2 5 5·0m A line lymphoid cells. In other words the immune status deteriorates with repeated exposure to antigen.

(b) The transfer of hyperimmune serum to normal CBA mice does not heighten their reactivity toward A line skin homografts on the contrary their reactivity is reduced (« enhancement »)

(c) The injection of hyperimmune serum into normal CBA mice counteracts the effect of injecting soluble A-line antigen. This effect still obtains when antigen and immune serum are injected by separate routes and on separate occasions two days apart so that it is almost certainly not due to a direct interaction between immune serum and antigen.

(d) Hyperimmune *cells* have no power to inactivate A line antigen when the two are mixed and exposed to each other for 90 min at 23

(e) The level of immunity achieved by the adoptive transfer of hyperimmune lymphoid cells is very low—a result entirely in keeping with the low level of active immunity (a)

(f) There is some evidence yet to be confirmed that the level of immunity produced by procedure (e) is still further reduced by the transfer of hyperimmune serum with the hyperimmune cells

The simplest interpretation of these results is in terms of a mutual antagonism between a sensitivity which has a cellular basis and an « enhancement » secured by some factor presumably an antibody that is present in hyperimmune serum. It is evident from result (a) that contrary to the prevailing belief mice which have at some time been highly sensitive (i e have a high degree of transplantation immunity) can still be « enhanced » in the special sense that their level of sensitivity can be made to decline The results of (c) make it highly unlikely that the hyperimmune serum procures enhancement by reducing or abolishing the sensitizing action of A line antigen. Its effect may still be due to some combination with antigen for example the epithelial cells of the A line skin homograft used to test for sensitivity might be coated with antibody in such a way as to make them less vulnerable to the action of sensitized cells In so far as it construes enhancement as the consequence of some action upon the target of the homograft reaction this interpretation resembles Kaliss's [11] rather than

our own (Billingham *et al.*[11]); our own was based upon the erroneous belief that mice once sensitive cannot be enhanced. The fact that the adoptive immunity produced by the transference of hyperimmune lymphoid cells is so feeble (c) is fully intelligible in the light of result (a).

The failure of hyperimmune lymphoid cells to absorb or inactivate antigen (d) distinguishes them sharply from regional lymph node cells which have been sensitized by skin homografts (Berrian and Brent, *Ann. N.Y. Acad. Sci.*, 73, 654, 1958). The distinction may be important.

DISCUSSION

OWEN: Relative to your suggestion that the T and H antigens are under the control of the same genes which may stamp their specificities on different molecular carriers, it seems to me that our only direct evidence on this point is on a single chromosome region, the H-2 region in the mouse. I wonder whether you might be inclined to modify your generalisation to say that, while there probably are loci which act in both of these capacities—perhaps the H-2 is an example—there may very well be other genes whose specificities are reflected in either H antigens or T antigens, but not in both. Even with regard to H-2 in the mouse, I wonder what *direct* evidence there is that these two types of antigens actually share a common specificity: are there, for example, evidences that a preparation of T antigens will absorb out anti-H activity from an antiserum or inhibit its action? I understood that you ruled out such a basis for the very interesting counteraction of the effects of T antigens by hyperimmune serum in the skin transplantation system.

MEDAWAR: I will do my best to answer those two questions, though actually Dr. Gorer is better qualified to do so. It is true that a full analysis of the ability of a particular allele to produce a transplantation antigen on the one hand and an antigen producing humoral antibodies on the other hand has been done only for the H-2 locus, but I think I am right in saying—Dr. Gorer will correct me if I am wrong—that it has also been studied in respect of the H-1 locus. But knowing from Professor Krohn's work how very many gene loci are involved in skin transplantation reactions in mice, it does indeed seem quite possible that some of these loci may be concerned purely with transplantation immunity reactions and may not be recognisable as H antigens at all. As for direct evidence that H antigens and T antigens share common determinant groups: as Dr. Owen says, if they did so one would expect T antigens to be able to absorb the humoral antibodies produced by

H-antigens. Hildemann and I made attempts to find out if T-antigens could absorb humoral antibodies and our results were all negative. This is by no means conclusive because if the T antigens have, for example, lipid attachments, it is possible that the determinant groups associated with the mucoid element are simply not accessible for the absorption of antibodies—they may be masked

HAŠEK I think perhaps that animals other than the mouse can be useful for showing that tissue antigens produce humoral antibodies and transplantation immunity at the same time. Chickens are excellent producers of free serum antibodies and one can recognise individual antigens by transplantation methods

MEDAWAR I agree with Dr Hašek. Dr Haddow and Dr Kozelka many years ago tried to find out if the humoral iso-antibodies produced by grafting in chickens would destroy *homografts* and I think they found no correlation between humoral antibody formation and transplantation imunity However I am speaking from memory

SIMONSEN There are three points I would like to raise in connection with this very stimulating paper *Firstly* when Dr Medawar states that the cell free antigens do not induce transplantation tolerance I would like to know if this *might not be because in making your preparations*, you actually destroy some of the antigens while others are retained in fully active form In order to induce tolerance you have of course got to induce tolerance against *all* the relevant antigens. In order to check this possibility I would like to know if you have tried to repeat your attempts to induce tolerance using a pair of co-isogenic strains because in that situation only one antigen should be active and therefore the failure to induce tolerance in that system would be very significant which I do not think is true of systems studied so far

Now another question is you discussed this remarkable discrepancy between the duration of the immunity produced by orthotopic skin grafts on the one hand and by intraperitoneal injections of lymphoid cells on the other hand Now could this not be the consequence of a graft versus-host reaction ? When you immunize by living lymphoid cells, they are almost certain to settle in the lymphoid tissues of the host and by doing so they possibly stimulate their immediately neighbouring host cells to antibody formation. But on the other hand they may also in the first day or so exert a reaction of their own against the very cells that are stimulated by their own antigens, and it may be that the antibody forming host cells are so impaired by this interaction

that they are not able to proliferate and therefore maybe die off during the first subsequent division.

Another point in this same connection is have you tried to investigate the duration of the immune response after injecting the lymphoid cells not intraperitoneally but intracutaneously because that would be more comparable to the situation of a skin homograft ?

A fourth point which occurs to me, is that in these experiments in which you hyperimmunise your CBA mice and find that the immune response is diminished in comparison to a single immunization would not that again fit in with a graft against-host interpretation ?

MEDAWAR Dr Simonsen s first question was about a possible reason for the failure of cell free antigens to induce tolerance. It is indeed possible that we destroyed some of the antigens in the course of preparation and, as Dr Simonsen says one could only get tolerance if tolerance is secured on behalf of *all* the relevant antigens However Brent and I are still inclined to believe that our failure is due to the fact which the work of Smith and Bridges and of Mitchison has brought out so clearly namely that to produce tolerance one must have *chronic* antigenic stimulation This seems to us to be the simpler interpretation.

Now as to the possibility that these hyperimmunized animals owe their properties to the fact that they may be involved in a graft against host reaction When one hyperimmunizes a CBA mouse with A line lymphoid cells the A line cells should have had no chance to counter attack the tissues of the host *unless* there were already some pre-existing state of enhancement Dr Simonsen s interpretation does not account for the enhancing properties of the immune serum taken from these hyperimmunized animals the hyperimmune serum has enhancing properties and also has the power to counteract the effect of injecting cell free antigens. One other question Dr Simonsen asked was whether the intracutaneous as opposed to the intraperitoneal injection of lymphoid cells is followed by a rapid decay of sensitivity The answer is yes.

BRENT As far as the question of inducing tolerance with antigenic extracts is concerned I think it very likely that some of the antigens *are* inactivated by our extraction procedures, as the work which Berrian is reporting later tends to suggest. But it is rather strange that on no occasion have we found any indication of even a partial prolongation of graft survival If some of the antigens were present in the extract—and we know they are, from immunization procedures—one would perhaps expect to see some slight prolongation of graft survival Another point concerns the question raised by Dr Simonsen on the graft versus

host reaction as a possible interpretation of the results of hyperimmunization by foreign lymphoid cells. The fact is that shortly after the first immunizing injection we do get a very strong transplantation immunity as can be shown by testing the animals with skin homografts three to five days after the injection

What happens to this immunity once it has been elicited?

What happens to the cell which in fact has been sensitized and which obviously has *not* been exterminated as a result of graft-versus-host reaction? I think that the fact that one gets a strong transplantation immunity reaction soon after the first injection of foreign lymphoid cells is pretty good evidence of the survival of host cells and that the decay of immunity must have some mechanism other than the graft against host reaction

SIMONSEN The problem should perhaps be studied by immunizing your CBA mice with CBA/A hybrid lymphoid cells.

Some recent data on the H 2 system of mice

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Having read some of the papers to be presented at this conference it occurred to the writer that it might be of interest to present some of the more recent data on the H 2 system. The latest published paper is that of Snell[7] but a great deal of serological data is in papers by Hoecker, Pizarro and Ramos[6] Amos[2] and Gorer and Mikulska[4] which have been submitted for publication but not yet appeared. No attempt will be made to review the literature most earlier references may be found in Gorer[3]

Crossing over within the H 2 system has now been detected several times. We have observed three examples. Allen[1] certainly detected one example and may have detected two. Snell[7] appears to have found a cross-over between $H\text{-}2^d$ and $H\text{-}2^q$ and perhaps one between $H\,2^k$ and an unknown H 2 type. Hoecker informed the author some time ago that he and his colleagues had found six cross-overs between D and K in the F_1 between H 2 and $H\,2^b$. Our data and theirs indicate a frequency of recombination between D and K in excess of one per cent. Recently Amos has found a cross-over in a different genetic situation (personal communication). We have been able to identify four dissociable loci DCVK. Work by Hoecker suggests the order DCVKH with the gene for brachyury (T) being adjacent to H. However his studies are not yet complete.

It is apparent that crossing-over must contribute appreciably to the polymorphism of the H-2 system. If we include cross-over combinations the number of pseudo-alleles known to exist exceeds twenty. Of these $H\,2^b$, $H\,2^d$ and $H\,2^k$ occur in unrelated stocks far more frequently than can be accounted for by chance and Gorer and Mikulska[4] point out that they must be favoured by selection. Together with H they are used more than any other combinations and in this article we shall concentrate on an analysis of them.

The number of antigenic differences that can be found to distinguish different individuals will depend in part upon the delicacy of available techniques, the distribution between different organs and tissues, etc. Most of the antigens in the H-2 system have been detected by the human serum dextran technique but two new ones have been found by the cyto-toxic technique (antigens « D^k » and « K^b ») which will be discussed more fully below. So far as the H-2 system is concerned probably the most important limiting factor is the number of combinations available for study. A method for calculating the number of components for N systems has been given by Gorer and Mikulska[4]. A component shared by all systems would appear to be an auto-antibody and in order to be detectible it must be absent from one and shared by (n-1), absent from 2 and shared by (n-2), etc.

TABLE I. — *Categories of antigens to be anticipated in four H-2 combinations (H-2a, H-2b, H-2d and H-2k)*

Category 1	Category 2	Category 3
H-2a not expected	H-2 (ab) not expected	H-2 (abd) F
H-2b « B »	H-2 (ad) D	H-2 (abk) E
H-2d E[1]	H-2 (ak) K	H-2 (adk) C
H-2k « D^k »	H-2 (bd) not found	H-2 (bdk) not expected
	H-2 (bk) not found	
	H-2 (dk) not expected	

Combinations such as H-2 (ab) denote antigens shared by H-2a and H-2b but absent from the other two.
Capital letters denote known antigens.

The four most generally used systems are H-2a, H-2b, H-2d and H-2k. Here we have three categories of components — 1) Those specific to one system e.g. H-2b. 2) Those shared by two which are designated as H-2(ab) etc. A similar notation being used for category 3. H-2 (abd) etc. Reference to Table I will show that fourteen components might be anticipated but four are listed as « not expected ». This is a consequence of the « dk » effect discovered by Snell[4]. It will be recalled that he showed that an F_1 between H-2^d and H-2^k was susceptible to certain A strain tumours and H-2a was called H-2^{dk} for a few years. In serological terms this indicates that no antigen should be present in H-2a that is absent from both H-2d and H-2k. Gorer and Mikulska[4] have presented circumstancial evidence to suggest that this is due to the fact that the H-2a resulted from a cross-over between H-2d and H-2k. It will be seen that no H-2a specific antigen is to be anticipated. In category 2, H-2a should not share a component with H-2b that is absent from *both* H-2d and H-2k. It is clearly a gross contradiction of

the « dk » effect if H-2d and H 2k shared an antigen that was lacking in H 2a and similar arguments apply to an H 2(bdk) component in category 3 It will be seen that under these special circumstances only 10 components should be anticipated instead of 14. In fact 8 have been found both missing components belonging to category 2 H-2(bd) and H-2(bk) There is no theoretical reason why both should not exist but if they are subsequently found they will probably be found to be weak components

TABLE II — *The dissection of antigen « B » by the employment of cross-over stocks*

Parental types	*Cross-over type.*	*Antigens* ( )			
H 2d / H 2b	H 2g	D^b	—	V	K^d
H 2a / H 2b	H 2h.c.	D^d	O	—	K
H 2a / H 2b	H 2i	D	O	V	K^b

( ) For a more complete list of antigens see Table 3.

New H-2 combinations may arise by mutation or crossing-over or the number available may be increased by the importation of new stocks. We have no certain knowledge of mutations within the H-2 system and Table II shows how « antigen B » or H 2b was subdivided by the study of cross-overs. The first of these arose in the F_1 (C57BL by BALB/c) the second and third in the F_1 (C57BL by A) The new combinations have been called H-2g H 2h and H 2i respectively and their corresponding stocks H 2G H-2H and H-2I Antigen F is not included in the table since all three parents are homozygous for it and for similar reasons both E and F are omitted from H 2h and H 2i. Antigen V was first detected in connection with H 2I. Previous studies had led us to believe that A anti-C57BL contained a « pure anti B » but certain samples of it agglutinate the red cells of H 2I mice. Absorption with the liver of H 2I mice did not abolish agglutination of C57BL, H 2G or H 2H red cells. The remaining agglutinin was called anti D^b Liver of C57BL and H 2G removed all agglutinins whereas that of H H left an agglutinin for H 2I red cells and the residual agglutinin has been called anti V Anti-K^b was found in H 2H anti-C57BL. It is doubtful if K^b is present on C57BL red cells and it was detected by the cytotoxic test. In any case it can be differentiated from V because H 2G has V but lacks K^b Further facts concerning the cross-overs may be obtained from the paper by Gorer and Mikulska[4] Here would stress the importance of cross-over combinations in the dissec

TABLE III — *Antigenic analysis of five pseudo-alleles*

H 2 Symbol	Antigens												Some typed strains
All	A	C	D	E	F	H	J	mK	M	N	V	Y	G I Q S	Some typed strains
a	A	C	D	E	F	H	J	K	M	N	—	Y	— — — —	A
b	—	—	D^b	E	F	—	—	K^b	—	N	V	—	— — — —	C57BL, C57L, 129LP STA
d	—	C	D	E^d	F	H	J	—	M	N	—	—	— — — —	BALB/C DBA/2, C57BL/Ks, YBR
k	A	C	D^b	E	—	H	—	K	—	—	—	Y	— — — —	C3H, CBA AKR, C57BR, STB 101
g	—	—	D^b	E^d	F	?	n.t.	—	—	N	V	n.t.	— — — —	H 2G
h	A	C	D^b	E	F	H	—	K	—	?	—	n.t.	— — — —	H 2H
i	n.t.	C	D	E	F	n.t.	n.t.	K^b	M	?	V	n.t.	— — — —	H 2I
r	n.t.	?	—	—	?	H	n.t.	—	n.t.	n.t.	n.t.	—	G I — —	A.CA, B6M
q	—	C	—	E	F	—	—	—	M	—	—	n.t.	— — Q —	DBA/1 C/st
s	n.t	C	—	E	F	H	n.t.	—	n.t.	n.t.	n.t.	—	G — — S	A.SW

— antigen absent ? = result of test ambiguous. n.t. = no test reported.
The new antigens AJMN from Amos GHIY from Hoecker et al.; D^b V K^b from Gorer and Mikulska
For further data on IR strains see Snell

tion of antigenic components that previously appeared to be single entities unique for a given H 2 combination. Similar studies may well show « D^k » and « E^d » to be equally complex

Some of the data obtained by Hoecker and Amos are presented in Table III It will be seen that the introduction of fresh H 2 combinations has revealed a considerably increased complexity in some of the original categories of components (see Table IV) Thus the

TABLE IV — *Extension of antigenic categories 2 and 3*

H 2ad from D to DJM
H 2ak from K to AKY
H 2abd from F to FN
H 2abk from E to ?
H 2adk from C to CH

H 2(ad) has antigens D J and M. H 2(ak) has AKY In category 3 antigen E remains surprisingly inviolate. Unfortunately it has not separated from K by crossing-over Of the remaining category three components H 2(abd) has F and N and H 2(adk) C and H Details of the discovery of these new components cannot be given but the principals are the same in all. The original four H 2 pseudo-alleles were either C+H+ or C—H— In order to distinguish the components the minimum requirement is either a C+H— or a C—H+ mouse Actually both exist. In addition to the antigens listed there are a number that await classification and some of these will certainly be members of the H 2 system. At present H 2a appears to have more components than other combinations. The A strain has red cells that are very sensitive to iso-agglutination and the investigation of the H 2 system began with the A strain. Recently Amos has found that C58(H 2k) and YBR (H 2d') have red cells of comparable sensitivity and those of strain F/St(H-2n) have remarkably reactive cells Had any of these been used as the starting point of investigation their respective H 2 types might have appeared to be the most complex. For reasons given above we must realise that symbols such as « D^k » or « Q » may denote a large number of antigens It is therefore very uncertain that H-2 combinations differ at all widely in the number of components they contain

So far 18 H-2 combinations have been sufficiently investigated to merit a symbol These occupy the alphabet from « a » to « s » with the astonishing omission of H 2o Strains A.SW A.CA and DBA/1 are fairly widely used and their H 2 types are included in Table III Most of the strains listed have been mentioned in previous publications It may be of interest to point out that strain 101 is H 2k It is generally stated that the ST strain is H 2k and we were therefore very surprised to find some ST mice we were typing for Dr Morten Simonsen were H 2b He informed us that they had two sub-strains of ST STA and

STB Unfortunately STA happens to be H-2b and STB is H-2k Doubtless the latter was sent to America Snell[7] has obtained a great deal of interesting information about H-2 types in some heterogeneous stock. For example the Swiss mice gave their name to strain A.SW and H-2s and Snell has found that they also carry H-2b and H-2d

It is undoubtedly advantageous to use strains of known H-2 type in immuno-genetic studies and it is advisable to have the types confirmed when possible. The discovery that the ST stock was composed of two sub-lines of different H-2 type came as a complete surprise The writer knows of more than one instance in which well known strains did not have the expected H-2 type. In one laboratory some C3H mice were found to be a mixture of H-2b and H-2k In this case it was established that a contamination had occurred. In other cases it has not been possible to establish the cause of the deviation It is only possible to say if a strain conforms to certain well established types such as H-2a, H-2b H-2d and H-2k and in some laboratories H-2s, H-2q or H-2f. If a strain does not belong to any of these types it may be typed for certain antigens but its precise classification would demand a major research project.

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DISCUSSION

OWEN I would like to consider briefly a reservation that may be relevant to part of the symbolic complexity suggested by Dr Gorer for the H-2 antigens. Table I illustrates a simple kind of test pattern frequently encountered We shall take this for the moment as representing the reactions of a set of red cell samples from four different individuals each tested against an antiserum produced by injecting the cells of sample 1 into a rabbit The cells of all four individuals react to this antiserum When the serum is absorbed by the cells of individual 1 against which the antiserum is directed all the antibodies are removed none remain in the absorbed fluid to react with the cells of any of the four individuals Absorption by the cells of individual 2

removes the antibodies with which these cells react, but leaves reactions for the other three individuals and a similar situation is found for individuals 3 and 4

As a symbolic representation of this simple test, the serologist would be likely to assign a letter say C, to an antigen recognized by antibodies in this antiserum and common to the cells of individuals 1 and 2 but absent from the cells of individuals 3 and 4 Thus absorption with either cells 1 or 2 removes anti-C from the antiserum and the cells of individual 2 fail to react after such absorptions Absorption with cells of individuals 3 or 4 leaves the anti-C antibodies in the reagent because these cells lack C. Similarly one might discern an antigen D common to individuals 1 and 3 and absent in the other two and an antigen E found in 1 and 4 Individual 1 therefore, becomes CDE individual 2 is C, 3 is D and 4 is E. Of course, this might be only the beginning of a complete analysis of this system

Figure 1 shows a test system with a distribution of reactions identical to that just discussed It throws a different light on our picture because, in this case, the chemical specificities on which the reactions are based are known. Landsteiner[11] by combining simple haptenic groups with protein carriers was able to discern serological reactions that depended on known chemical additions to the immunizing and test antigens. In the instance illustrated metanilic acid diazotized to horse serum was used as the immunizing antigen and this hapten and other similar ones were diazotized to chicken serum for the test antigens, and to sheep red cells stromata for absorptions You will note that the antiserum is not, in fact completely specific for the chemical grouping that directed antibody formation it cross-reacts with haptens in which the sulfonic acid group is in the ortho rather than in the meta position or in which arsenic or benzoic acid substitutions are made for the sulfonic acid grouping in the meta position. When such an antiserum is absorbed by this set of related materials, antibody cross-reactive with each of these haptens is removed to leave residual antibodies still reactive with the others. Only the hapten that directed the formation of this heterogenous antibody population reacts with all its fractions.

Comparing table I and figure 1 we can discern a basis for serious reservation concerning the relation between the patterns of cross-reaction of antigens and details of similarity or difference in their structure. The symbolism CDE for cells of type 1 in table I suggesting that the cells include three antigens, each shared with one of the other three individuals, cannot be related to the true character of the immunizing cells or their antigens The symbols actually stand for fractions of *antibody* in a heterogenous molecular population In figure 1 we note a fraction

TABLE I

Absorption analysis of an antiserum to red blood cells of individual 1

Test cells.	Serum unabsorbed	Serum absorbed by			
		1	2	3	4
1	+	O	+	+	+
2	+	O	O	+	+
3	+	O	+	O	+
4	+	O	+	+	O

TABLE II

Antimetanilic Acid Azo-Horse Serum

Test antigens (— azo — chicken serum)		Unabsorbed	Absorbed by azostromata			
			(1)	(2)	(3)	(4)
(1) Metanilic	NH_2 / SO_3H	+	O	+	+	+
(2) o-Aminobenzene sulfonic	NH / SO_3H	+	O	O	+	+
(3) m-Aminobenzene arsenic	NH / AsO_3H	+	O	+	O	+
(4) m-Aminobenzoic	NH / COOH	+	O	+	+	O

of this population peculiarly reactive with metanilic and o-aminobenzene-sulfonic acid removed by absorption with either of these haptens this antibody fraction could be symbolized as anti-C. Similarly we can discern antibody fractions that could be symbolized anti D and E. But these have no necessary relation to any detail of structure in the test antigens. E, for example cannot stand for a carboxy substitution in the meta position because a sulfonic acid substitution also reacts with it

in fact, it was a sulfonic acid that induced the anti E antibodies Neither can it stand simply for a meta substitution because a meta substitution of arsenic acid does not produce the specific reactivity to which we might assign the symbol E The symbols we have used represent only a shorthand way of saying that the cells of individual 2 are *similar* in some respect to those of individual 1 because they react with antibodies induced by 1 Individuals 3 and 4 also have something in common with 1 but they are different from each other and from 2 so we arrive at different symbols for them D and E respectively But this does not mean that the cells of individual 1 which induced all these antibodies therefore include three separate and distinguishable antigens. A single simple hapten would be subject to equally complex representation on such a basis, and its apparent complexity would be limited only by the number of other haptens at hand sufficiently similar to cross-react with separable fractions of the complex antibody population it induced and by the complexity of that population It may be, therefore, that the *genic* and *antigenic* complexity of H 2 is less than would appear from the symbolic representation Dr Gorer has given

GORER I hope you are right. The serological behaviour of these antibodies is not like that of cross-reacting antibodies of that kind There is no cross-reaction as far as I know between E and K. They are immunologically quite different from one another and not like you would get with related sulphonic acid etc. So I am a bit pessimistic about the whole thing I think the complications are genuine.

Antigen and antibody in transplantation immunity: Some problems of investigation

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PART I

INTRODUCTION

Our interest in the search for a transplantation « antibody » stems from our earlier observations on kidney homografts in man Here we found that the rejection of kidney homografts occurred much more slowly in many patients than they did in the experimental animal [11] This appeared however to be a quantitative and not a qualitative difference probably related to the fact that these patients were chronically uremic and as such debilitated. Possibly therefore they were incapable of as « active » an immunologic response as normal healthy experimental animals. We found for instance [11] that the rejection of skin homografts occurred much later in chronically uremic patients than they did in normals The technical experience gained in renal homotransplantation in the human however was not completely lost since it enabled us to successfully transplant a kidney from one to another of identical human twins [12] Since that time transplantation of the kidney has been successfully performed in 11 such instances It seemed apparent however that any further work whose ultimate aim was successful kidney homotransplantation in man must be based upon a thorough understanding of transplantation immunity and upon success in modifying the rejection response of homotransplanted tissue

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In 1955 Hoigne and his collaborators[16, 17, 18] described a method for the identification in serum of « sensitivity » to allergens such as penicillin and sulfonamides. Addition of the specific allergen to serum of patients with clinical sensitivity to these allergens showed increased turbidity in a nephelometer Bollag[6, 7] applying this « zonal turbidity reaction » of Hoigne believed that he could demonstrate antibodies which were individual specific following the transplantation of skin between rabbits. Bollag's « antigen » was a watery extract of the donor tissue and could be extracted from any tissue of the individual donor We were unable to confirm these results in humans following the transplantation of skin In our experiments the Beckman spectrophotometer was used to measure turbidity and the recipient serum was mixed with an aqueous extract of white cells from the donor Other workers[9, 15] have also had difficulty in repeating the results of Hoigne with the nephelometric technique.

About this time we were stimulated by the results of Kurnick[21] who reported results of the treatment of disseminated lupus erythematosus by the injection of white cells homogenates The similarity between the pathology of disseminated lupus and the rejection of the homograft is suggestive. It seemed possible that a circulating antibody might be responsible for the lesions of disseminated lupus as well as for the specific « antileukocyte » factor resulting in the LE phenomenon We reasoned somewhat naively that it was possible that Kurnick's results might be explained by the combination of circulating antibody with injected white cell antigen, thus diminishing the amount of isoantibody available for reaction with body tissues and white cells. Kurnick himself attributed his results to the « DNase inhibitor » content of the white cell homogenate. We attempted therefore, to apply this technique to prolonging life of skin homografts by multiple subcutaneous injections of donor white cells This was wholly unsuccessful and in retrospect there are many reasons why they should be so First of all we had no idea about the potency of our « antigen » Secondly we were ignorant of the necessary time relations between grafting and injection of antigen and finally there was no real evidence indeed that adequate circulating antibody existed to combine with injected antigen We therefore repeated the experiments, this time injecting 50 cc of whole blood (so that donor white cells might not be destroyed or modified by previous processing) at daily intervals for seven days beginning some 10 or 12 days before the placing of homografts. Some suggestive evidence was obtained that we could accelerate the rejection of grafts from the donor whose white cells had been injected in this way when compared with control grafts not previously modified by injection of donor white cells. At this point in our work Billingham and co-authors[3] reported that rejection of skin

grafts might be accelerated by prior injection of a water soluble extract of spleen cells from the same donor strain. This water-soluble clear extract was thought to contain the « transplant antigen » in the DNA fraction and in amounts approximately 1/100 that of whole white cells when compared per mg of nitrogen. It seemed to us an admirable preparation for it afforded the opportunity to correlate the biologic activity with some of the more classic immunologic techniques employing the lysate as a soluble antigen.

According to Billingham's earlier publication the antigen responsible for transplantation rejection was to be found in the DNA fraction of the lysate. It was of some interest therefore that we read of Seligman's observation [31] that in disseminated lupus a globulin could be isolated from the serum of patients with a positive LE preparation which could be precipitated with purified DNA prepared from calf thymus. This anti DNA globulin could be separated from the precipitated antigen antibody complex by the use of DNAse and when added again to normal white cells produced the characteristic LE phenomenon. This seemed a further parallel between those « antibodies » responsible for disseminated lupus erythematosus and those possibly responsible for homograft rejection. By the use of a fluorescent antibody technique Bardawil [4] has demonstrated the fixation of fluorescent globulin to cell nuclei in the serum of patients with disseminated lupus and a positive LE phenomenon. This seemed an opportunity to test the possibility of « exhausting » *in vivo* the antibody in the serum of patients with lupus by the injection of antigen in the form of DNA. A DNA histone preparation made from calf thymus was injected in increasing amounts totaling 34 ml (approximately 34 mgm of DNA) over a period of five days in a patient with disseminated lupus who had a positive LE phenomenon as well as a positive fluorescent globulin fixation. Both of these tests were reversed following the injection of this material and there was coincident improvement of the patient although it is not possible to attribute this directly to the injections. Twenty-six days after the cessation of injections the LE phenomenon became positive again. It thus seemed possible that a soluble preparation containing nuclear material might indeed modify a biologic phenomenon associated with « auto-antibodies ».

Since in the human and larger animal it is not feasible to use a lysate of splenic tissue to study the homograft reaction we have employed circulating leukocytes which have also been shown to accelerate the rejection of skin homografts. Using the soluble antigen from the lysate of white blood cells harvested from buffy coat of peripheral blood we have explored the possibility of demonstrating circulating antibodies by conventional immunologic methods using as the source of antibody the

the fashion of Billingham[5] The reaction was species specific although not specific for the individual donor We still, however were unable to convincingly demonstrate this with the homograft reaction since the precipitin reaction between donor lysate and recipient serum could be demonstrated in only 50 per cent of animals following the sloughing of multiple large skin grafts to rabbit ears. Similarly the immunization of rabbits with rabbit white cells produced inconsistently positive precipitins. The technique of tanned red blood cell hemoagglutination had been shown to be a more sensitive reaction for the demonstration of the serum antibody in lupus[12] We attempted to demonstrate the homograft antibody with tanned red blood cells after the method of Stavitsky[24, 25] using as antigen fixed upon surface of the tanned red cells the lysate of donor white cells. Again the results were equivocal since while positive tests did occur the controls were also positive in many instances. It is thus appeared that living nucleated cells from a heterologous species could produce in the serum of the recipient immunized with these cells precipitins against a soluble extract of the cells. Since this soluble extract had been shown to accelerate the rejection of skin grafts, these data suggest that there might be a common antigen. However although the reaction was species specific it was nevertheless immunization against a heterologous antigen From a biologic standpoint however it did not seem reasonable to assume that the transplantation antibody was necessarily a precipitin or a hemoagglutinin Certainly however part of the system must be a cytotoxin. Gorer[14] has demonstrated that mice immunized by the transplantation of tumor may develop in their serum antibodies which are cytotoxic for normal spleen cells of the donor This cytotoxicity is demonstrated by the uptake of eosin or trypan blue when donor cells are incubated in a system containing serum of the immunized recipient and complement We found the serum of rabbits immunized against whole human white cells or the lysate from whole human white cells to contain antibodies strongly cytotoxic for leukocytes from the donor species but not for other species. Thus at least for a heterologous system a cell free lysate capable of producing accelerated rejection of skin was capable of producing both precipitating and cytotoxic antibodies. In the human there is good evidence that in a homologous system repeated infusions of white cells from different donors may produce in the recipient anti white cell agglutinating antibodies[8] However even in this system the antibodies are not individual specific but may be demonstrated by the use of any normal human white cells as antigen. In the sense however that the recipient's own white cells are not agglutinated by these antibodies, they are isoantibodies

Discussion

The results presented here demonstrate that a cell free extract of living cells can be used to produce precipitating antibodies in the recipient animal Of more importance, however to the problem of transplantation of tissues is the fact that this cell free extract also produces antibodies which are cytotoxic for leukocytes of the donor species but not of other species Although this cell free extract used was prepared from a heterologous donor extracts of homologous cells prepared in the same fashion have been demonstrated to accelerate the rejection of skin homografts. It seems probable that heterologous lysate would have the same biologic activity for skin heterografts. Allgower and his colleagues[3] obtained essentially similar results with a saline extract of heterologous skin They too were able to demonstrate precipitin reactions between the serum of animals immunized with such an extract and the extract itself and by the tissue culture technique were able to demonstrate a cytotoxic effect of such immune serum against donor skin cells.

Following the rejection of multiple skin homografts to rabbit s ears, however only 50 per cent of our animals developed positive precipitin tests, when the plasma was incubated with lysate or donor white cells In four experiments no cytotoxic effect was found against whole donor leukocytes. While the significance of these figures is questionable, it is important to note that none of the autografts developed precipitating antibodies, nor was the control plasma positive in any of the experiments In Allgöwer s experiments neither cytotoxic effect nor precipitins were demonstrated following immunization with homologous skin extract and the transplantation of whole homologous skin.

That the positive precipitin tests in our recipient animals were not due to contamination with donor white cells is evidenced by the fact that homologous leukocytes in large numbers placed beneath an autograft never developed positive precipitins Contamination with red blood cells was not a factor since lysate of homologous red cells alone gave negative results with sera which were positive with white cell lysates. Some red cell contamination of the harvested leukocytes was inevitable But while rabbits immunized against human leukocytes did develop agglutinins for the red cells of the donor (human) red cell agglutinins alone were not cytotoxic for donor white cells, since no cytotoxic effect could be demonstrated when leukocytes from an A negative human were incubated with high titer anti A sera. Since Coombs[14] has demonstrated the presence of red cell antigens upon human leukocytes it was important to rule out this possibility

the fashion of Billingham[8] The reaction was species specific although not specific for the individual donor We still, however were unable to convincingly demonstrate this with the homograft reaction since the precipitin reaction between donor lysate and recipient serum could be demonstrated in only 50 per cent of animals following the sloughing of multiple large skin grafts to rabbit ears. Similarly the immunization of rabbits with rabbit white cells produced inconsistently positive precipitins. The technique of tanned red blood cell hemoagglutination had been shown to be a more sensitive reaction for the demonstration of the serum antibody in lupus[13] We attempted to demonstrate the homograft antibody with tanned red blood cells after the method of Stavitsky[26, 27] using as antigen fixed upon surface of the tanned red cells the lysate of donor white cells. Again the results were equivocal since while positive tests did occur the controls were also positive in many instances. It is thus appeared that living nucleated cells from a heterologous species could produce in the serum of the recipient immunized with these cells precipitins against a soluble extract of the cells. Since this soluble extract had been shown to accelerate the rejection of skin grafts these data suggest that there might be a common antigen. However although the reaction was species specific it was nevertheless immunization against a heterologous antigen. From a biologic standpoint however it did not seem reasonable to assume that the transplantation antibody was necessarily a precipitin or a hemoagglutinin Certainly however part of the system must be a cytotoxin. Gorer[14] has demonstrated that mice immunized by the transplantation of tumor may develop in their serum antibodies which are cytotoxic for normal spleen cells of the donor This cytotoxicity is demonstrated by the uptake of eosin or trypan blue when donor cells are incubated in a system containing serum of the immunized recipient and complement. We found the serum of rabbits immunized against whole human white cells or the lysate from whole human white cells to contain antibodies strongly cytotoxic for leukocytes from the donor species but not for other species. Thus at least for a heterologous system a cell free lysate capable of producing accelerated rejection of skin was capable of producing both precipitating and cytotoxic antibodies In the human there is good evidence that in a homologous system repeated infusions of white cells from different donors may produce in the recipient anti white cell agglutinating antibodies[9] However even in this system the antibodies are not individual specific but may be demonstrated by the use of any normal human white cells as antigen. In the sense, however that the recipient's own white cells are not agglutinated by these antibodies they are isoantibodies

Discussion

The results presented here demonstrate that a cell free extract of living cells can be used to produce precipitating antibodies in the recipient animal Of more importance, however to the problem of transplantation of tissues is the fact that this cell free extract also produces antibodies which are cytotoxic for leukocytes of the donor species but not of other species. Although this cell free extract used was prepared from a heterologous donor extracts of homologous cells prepared in the same fashion have been demonstrated to accelerate the rejection of skin homografts It seems probable that heterologous lysate would have the same biologic activity for skin heterografts. Allgöwer and his colleagues[3] obtained essentially similar results with a saline extract of heterologous skin They too were able to demonstrate precipitin reactions between the serum of animals immunized with such an extract and the extract itself and by the tissue culture technique were able to demonstrate a cytotoxic effect of such immune serum against donor skin cells.

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If one accepts the possibility that circulating « antibodies » may be responsible for the rejection of homografts and that the cell-free lysate contains an « antigen » which may be responsible for transplantation immunity one might expect to demonstrate by some immunologic technique such « antibodies » in the serum of animals immunized against homografts if such a lysate were used as antigen in the immunologic reaction. Several factors may be responsible for a lack of consistent demonstration of such a reaction in our experiments and those of Allgower 1) The « antibody » may not be a precipitating or cytotoxic antibody although it certainly seems to be in the heterologous reaction 2) As the results of Algire suggest the rejection of a homograft may *depend upon the presence of whole sensitized mononuclear cells.* Algire's results however do not rule out the possibility that the sensitized mononuclear cell may simply contain the « antibody » in concentrated form This is suggested by the fact that heterograft antibodies may pass the millipore barrier and cause rejection of heterografts without the presence of sensitized cells from the recipient. It is suggested also by the reports of successful transfer of sensitivity to simple chemical compounds (for which no circulating antibodies have been found) after the disintegration of sensitized blood leukocytes by sonic vibration Similarly iso-*antibodies produced by the transplantation of tumors in resistant strains* will enter the millipore chamber[3] From the histologic standpoint too it would not appear that whole cells were an integral part of the transplantation rejection In the accelerated « second set response » the early part of the rejection response is not characterized by the presence of recipient round cells. Leukocyte antigens are found in small amounts in *normal human serum*[15] *and it is possible that the difference between* serum and whole cell « antibody » too may be a question of concentration. Since the rejection of a small piece of homografted skin would require only small amounts of circulating antibody concentrated at the donor site the circulating blood might be easily cleared by absorption at this site of any circulating factors. In this sense the size of the antigenic stimulus in our experiments would be inadequate to demonstrate circulating antibodies by conventional techniques. This is suggested by the fact that small experimental skin grafts in our experience have never produced precipitins against lysate of donor white cells while in the one human instance in which large amounts of skin were repeatedly grafted such precipitins were demonstrable 3) The biologic similarity of the antigen to the host's antigenic pattern may also play a role Thus circulating antibodies are easily demonstrable following the rejection of heterografts whereas they are not with homografts. 4) Finally the failure to demonstrate circulating homograft antibodies may be due to the fact that such antibodies are absorbed by the donor antigen An

ingenious demonstration of the importance of this fact has recently been made by Feldman [12] who demonstrated the formation of antihost anti bodies by grafted marrow only after retransplantation of the marrow into the donor where there was no antigen from the original host to « absorb them »

PART II

It seemed obvious that the use of plasma or serum was not productive in eliciting *in vitro* evidence of transplantation immunity The following experiments were then performed. Rabbits were sensitized by two or more skin grafts and following the rejection of the last graft serum and plasma were taken from the recipient. Mononuclear cells were also obtained from the recipient by peritoneal irrigation and circulating leucocytes of the donor animal served as the antigen in the test system Both precipitin tests with serum and lysate of donor cells, and cytotoxic tests with whole cells of donor and recipient were done. The results here were also equivocal. We then reasoned that it was possible that if, as Gowans has stated the lymphocyte population is constantly recirculating, and that only a small proportion of these cells are « sensitized » it might be necessary to attract the sensitized cells into the peritoneal cavity for harvesting by a specific antigenic stimulus Using animals in which skin grafts had been rejected, we then injected the night before into the peritoneal cavity of the recipient a suspension of donor epidermal cells made by trypsinising pieces of skin With this method we have obtained strongly positive cytotoxic results in the last three experiments which were done. Interestingly enough the cytotoxic effect was clearly demonstrated only when recipient and donor cells were incubated without plasma or serum Indeed, the addition of serum seemed to inhibit the effect. This may possibly bear on the inhibiting effect on skin graft rejection seen following the intravenous administration of hyperimmune serum

We have also confirmed in the human the results of Brent presented at this conference in the elicitation of a positive skin test of a delayed type in animals which have been sensitized by skin grafts. We have had occasion to study a pair of twins one with severe kidney disease, the other healthy These twins shared 23 identical blood group antigens but did not appear physically monozygotic. The sick twin had retained a skin graft from the healthy twin but the healthy twin had rejected two skin grafts from his sick brother The rejection of the first graft was delayed to 23 days but both pieces of skin were characteristically and undoubtedly rejected Following the rejection

of skin by the healthy twin we were able to elicit a strongly positive and characteristic tuberculin type skin test by the intradermal injection of donor white cells. Donor red cells were slightly *positive (almost certainly due to contamination by white cells)* Autologous cells donor *plasma and cells from* non related donors were quite negative. It is of interest that in comparable cell dosage the response was much stronger on the arm on which the skin grafts had been placed although it was also positive on the contralateral arm Aqueous lysate from donor white cells was also positive. Lysate from cells of a non-skin donor showed a faint positive. We have not yet determined whether this was due to an artefact of preparation or possibly to the fact that degradation of individual specific antigen may give a cross-reaction

Discussion

It is interesting to speculate that possibly the inability to obtain circulating antibodies in homograft immunity may be due to the fact that as Feldman[12] has suggested they are adsorbed by donor antigens. It is equally possible, though hypothetical that the similarity of the donor and recipient transplantation antigens may allow anti-donor anti*bodies to be fixed loosely to recipient tissues as sessile antibodies There* is precedent for this in classic immunologic thinking If this were true it might account for the fact that heterologous or dissimilar antibodies may be found in the circulating plasma while homologous are not It may account for the fact that antigen antibody complexes injected intracutaneously can give delayed type sensitivity It may account for the paucity of round cells seen in the second set reaction Finally though we have only one observation it might account for the increase in *strength of the tuberculin type skin test with increasing proximity to* the site of the injected graft

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DISCUSSION

MOUREAU I would like to ask Dr Merrill if it is quite certain that it was the leucocytes as such which gave rise to the intradermal reactions in the twins or whether it could not have been a simple histamine phenomenon ?

MERRILL I think all we can say is that the controls that is white cells taken from non related individuals, showed no such reaction Whether the reaction has any specific relation to the transplantation

antigen I think remains to be seen as Professor Medawar pointed out But I think we can say at least in our system that it is specific for donor white cells and not for non related white cells.

VOISIN I would like to make one comment arising out of Dr Moureau's question as a control instead of autologous cells intradermally injected, I would have liked to have seen homologous cells Of course if the result had been positive we could always say that there were common antigens between the skin donor and the donor of cells. But if the reactions were negative then is that not the best control you can get ?

MERRILL I am sorry I did not make myself clear We do not have the slide showing the use of homologous cells but such controls were done they were negative on three occasions from three non related donors.

VOISIN That is the best control

MERRILL Yes.

BRENT I am of course fascinated to hear that Dr Merrill and his colleagues have been able to show a delayed cutaneous reaction with tissue antigens in the human being Judging from our experiments with the guinea pig may I suggest that it may perhaps be a little premature to conclude from a single experiment that the sensitivity is regional ? In the guinea pig we find—as others working on the tuberculin reaction have done before us—that a lot depends on the way in which the antigen is injected into the skin. There is often variability in the degree of inflammation in adjacent sites on the same animal

The other point which I would like to comment on is your finding that the fluorescent antibodies are not in your heterologous system concentrated very close to the nucleus I think it is true to say that there is no evidence at all that heterologous transplantation antigens are necessarily nuclear In fact there is reason to believe that they may be cytoplasmic as well as nuclear The concept of nuclear antigens is chiefly derived from work on homologous tissue antigens

I cannot say I have followed Dr Merrill's speculations concerning the absorption of antibodies by host tissues If antibodies are produced against homologous antigens, it does not seem very probable that they would be absorbed by the host's own tissue antigens

MERRILL I quite agree of course and my conclusions were identical with yours about the heterologous system

I think perhaps we have a little difference of opinion about the antibody I do not of course imply that the production of the antibodies which might be fixed loosely on recipient tissue is produced by or against the patient s tissue itself What I do mean to imply is the sort of thing suggested by Dr Owen that a specific antigenic stimulus may produce a variety of antibodies some of which are not necessarily specific for that tissue. Cross reactions like this certainly do occur but biologically the important thing is the reaction of the specific antigen with the specific antibody produced against it. And this is what I meant to show by my last slide which represents antibody fixed against non isologous tissue if you will, against homologous tissue in a position in which we know it does not cause clinical disease. Now with regard to the significance of our one experiment I must apologize, but I can only say in my defence that twins with 23 identical blood groups who reject homografts are rather rare.

MEDAWAR May I just ask Dr Merrill if his twins have been checked to find out if they are chimeras, perhaps asymmetrical chimeras?

MERRILL I am not quite sure. If they were chimeras they would be total chimeras at least blood group chimeras, because every blood group antigen they have that has been checked is shared and the pattern is quite different in their mother and father and one sibling

OWEN You do not find evidence that anti Kell reacts more strongly with the blood of one than with the other suggesting that part of the cells may be Kell-positive and part Kell negative, or any indication of that sort?

MERRILL I cannot answer that We of course did not do this ourselves. It was done in the blood-grouping laboratories of the Children s Hospital in Boston by Dr Fred Allen and he assured us that insofar as he could tell they were identical antigenically

OWEN The skin tests have been referred to by Dr Brent as delayed reactions. I was not quite clear from your presentation as to whether it may be possible that they were immediate wheal and flare anaphylactic reactions?

MERRILL I did not think it was necessary to make that point Of course we checked them immediately for 2 hours, 8 hours, 12 hours and 24 hours. They were perfectly classical delayed type reactions.

HYATT I think it is a good point to stress the magnitude of the differences involved when dealing with relatively standard animal strains and the patients particularly the chronically ill patients. For instance I wonder if Dr Merrill would indicate how long the period of observation has been on the skin graft because in the syngenesial plastic graft it could persist for a considerable time. I would be particularly interested in the globulin both the chemical and electrophoretic pattern of this chronically ill patient One point of clarification missed were the healthy patient's autologous cells processed similarly to the controls from the standpoint of the intradermal reading? I missed that point

MERRILL I had tried to make very strongly this very point that has just been suggested that the sick patient is unquestionably a different individual in regard to the development of transplantation immunity As matter of fact we did transplant a kidney in this individual simply on that basis. If we had not believed very strongly that this were true we would not have undertaken such a procedure so there is no question about that at all in my mind as we have explained many times. Now as to how long the skin has taken on the healthy individual I believe the first skin graft was placed in November or early December last year and it is still pretty good As to the last question cells from both patients were prepared for intradermal testing in an identical manner

MURRAY The skin graft has so far lasted for 86 days.

MERRILL The electrophoretic pattern on this individual I have not had an opportunity to observe yet I would be a little loth to attempt to interpret them but I can say that in a good many other uremic patients that have shown prolonged skin takes the electrophoretic pattern has been quite normal provided that they have adequate nourishment

SIMONSEN As to the cytotoxic antibody tests you performed in skin grafted rabbits you made the very interesting observation that immune cells from the peritoneal exudate when mixed with donor leucocytes gave a very strong reaction a + + + reaction as far as I can remember When the same immune cells, er [illegible] with supposedly immune plasma and the sam[illegible] [illegible]ve a very weak reaction I do not think [illegible] of this but I suppose that one of the th[illegible] [illegible]incing antibodies or something like th[illegible]

bodies would be effective on the efferent side of the antibody reaction. I would like to know if you have tried first to mix your donor cells with the supposedly immune plasma of the recipient, wash them again to remove the plasma proteins except of course the enhancing antibodies which should be fixed to the donor leucocytes and then to mix the donor leucocytes with immune cells of the recipient. Have you tried that?

MERRILL We have not tried it quite in that fashion and I have no explanation at the present time as to why we should have a weaker reaction with serum plus sensitized cells, but I think certainly that the type of explanation that has been offered here is consistent with other results.

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Some problems of induction of transplantation tolerance

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The study of tissue incompatibility process in homografting step by step led to the conclusion that tissue incompatibility is due to a peculiar immunological reaction transplantation immunity. Both experimental results and the lack of clinical success in homografting most tissues indicated that every individual in a normal population possesses practically a unique antigenic mosaic. Thus the results obtained in homografting confirmed the supposition of the immunologists speaking of an antigenic uniqueness of the individual at the very outset of the blood group study. Up to now there is no possibility that the living homograft might be deprived of antigenicity or transformed in such a way that it could be tolerated by the recipient. Another approach to overcoming tissue incompatibility is in suppressing the recipient's immunity to a foreign graft. Immunology from the beginning concerned mainly with producing or increasing immunity to infectious agents is faced here with a new problem motivated by practical application, i.e. that of removing the undesirable immunological manifestations in transplantation. *In this respect the phenomenon of acquired tolerance as a result* of embryonal adaptation to a foreign antigenic stimulus seems to be a promising line along which further research might be pursued.

The concept of acquired tolerance is based upon the findings made with dizygotic bovine twins. The observation of blood chimaerae in dizygotic bovine twins which are natural parabionts (Owen) led Burnet and Fenner [4] to an immunological interpretation of these facts. Medawar et al [1, 2] *showed that skin grafts took successfully in dizygotic* twins and thus laid the foundations of the concept of acquired tolerance which was further developed by them under experimental conditions (Billingham, Brent, Medawar [3]).

In our experiments we reproduced experimentally the synchorial anastomosis occuring in calf twins The method of parabiosis in chick embryos was used. From the immunological aspect chicks are splendid antibody producers and respond well to the partner's blood cells. Consequently individual antigenic differences in chickens can be demonstrated very well by means of serological tests as well as by transplantation. In experimental chicken parabionts we demonstrated both the immunological tolerance in an orthodox antigen antibody system and the transplantation tolerance. It was found that chicks which had been embryonic parabionts, were not capable, even in adult life, of forming antibodies against the partner's erythrocytes after repeated immunization (Hašek [13]) It was also found that skin homografts from the partner take in postfetal life (Hašek [14])

Both original methods of experimentally obtaining tolerant animal i.e. Medawar's intraembryonal injections and embryonal parabiosis are loaded with a high mortality rate among the experimental embryos A far simpler way of inducing tolerance in some species was found by administering large amounts of antigen soon after birth. We found that tolerance in chicks can be produced in over 50 % of the birds by a large dose of homologous cells after hatching In ducks it was found that the adaptive period for homologous cells is prolonged for several days after hatching Puza and Gomboš [28] produced tolerance in newborn puppies by exsanguination transfusion with the blood of an adult donor

Tolerance is maintained long into postfetal life, but it disappears in the majority of animals when long term follow up observations are made. In exceptional cases tolerance seems to persist for a very considerable period of time, the whole lifetime perhaps. In one case a skin homograft took well in one pair of 2 year-old parabionts In 12 out of 15 animals complete homograft tolerance persisted for 6-18 months in 2 animals it was maintained for more than two years and in one case it lasted as long as the animal lived, i.e. four years. A histological examination of skin grafts which were destroyed after a more extended survival shows that even here transplantation immunity response is developing Yet the very process of graft breakdown from the initial slight haemorrhagiae in the graft over the lymphocytes and plasmacytes infiltration up to the final massive infiltration and necrosis of the graft seems to require ample time for the development. Cannon [7] believes that only skin grafts from young animals are tolerated We are of the opinion, however that the failure of his experiment with respect to retransplantation of second-set skin grafts on chickens tolerating homografts exchanged between newly hatched chickens is due to an incom-

plete tolerance. In our experiments, it was found that in all ducks, which tolerated for a long period of time a skin homograft exchanged on the first day after hatching also the second set graft from the same donor was tolerated (Table I)

TABLE I — *Retransplantation of skin grafts in ducks with a long-term tolerance of the first skin graft, exchanged between newly hatched birds*

No of	1st graft survives (transpl. between one-day-old birds)	2nd graft survives (transpl between 4-week-old birds)
8	> 250 days	> 200 days

HETEROLOGOUS TOLERANCE.

We assumed that after we had succeeded in reproducing the state of natural parabiosis in calves with its immunological consequences it would be possible to induce the interspecific tolerance under experimental conditions It was not difficult to join two embryos of different species during experimental parabiosis. We observed transplantation tolerance in the following interspecific combinations duck-chicken turkey-chicken, pheasant-chick. *In contrast to intraspecific parabionts* in interspecific parabiosis only a very weak degree of heterologous tolerance was found *Only a moderately prolonged* survival of skin homografts from the partner in embryonal parabiosis was observed yet the grafts always regressed within 30 days at the latest (Hašek [14]). It was, therefore, necessary to consider the fact that heterograft incompatibility in tolerant animals might be due to other factors, such as nutrition or other effects as well. *Yet parallel experiments in which tolerance was* tested in antigen-antibody system showed that the ability to form antibodies was merely decreased in tolerant animals or they were able to produce antibodies in the normal way In parabionts between ducks and chickens it was found that in post-embryogenesis they are capable of forming antibodies against the partner's erythrocytes *though in a* lower titre when compared with the controls (Frenzl et al. [17]). These results were confirmed by further interspecific combinations (Hašek [14]). Many other serological studies of immunological tolerance to various antigens in different laboratories indicate that it is extremely difficult to produce tolerance to antigens substantially different from those of the recipient's body

It was possible, however *to induce a higher degree of heterologous* tolerance between some, in the zoological sense more closely related species *Thus skin goose heterografts placed on ducks intravenously*

inoculated by a suspension of isolated goose bone marrow cells within 20 hours after hatching survived almost three times longer than in prior untreated animals (Table II)

TABLE II — *Survival of goose skin grafts on newly hatched ducks*

	No of injected cells.	First macroscopic signs of destruction (days)	Total destruction (days)
Controls	—	15	17
	—	9	11
	—	9	11
	—	9	11
Injected birds	46 × 10⁴ i.v	32	42
	92 × 10⁴ i.v + 184 × 10⁴ s.c.	29	42
	92 × 10⁴ i.v + 184 × 10⁴ s.c.	29	42
	69 × 10⁴ i.v + 184 × 10⁴ s.c.	29	42
	46 × 10⁴ i.v	29	42
	46 × 10⁴ i.v	11	15
	46 × 10⁴ i.v + 20 × 10⁴ s.c.	21	30

However the majority of adult ducks to which isolated goose bone marrow cells were administered were not capable of forming hetero-agglutinins against goose erythrocytes after being immunized (Table III). In a part of animals the capability of antibody formation was completely suppressed.

TABLE III. — *Results of immunisation with goose blood in ducks following injections of isolated goose spleen and bone marrow cells*

	Titre of heteroagglutinins	
	after 1st immunisation.	after reimmunisation.
Controls	16	64
	128	512
	16	128
	32	256
Injected birds	0	0
	0	0
	4	2
	0	0
	2	2

Embryonal parabiosis appears to be more effective in conferring tole rance than a single injection of cells probably because a larger amount of antigenic material acting a relatively long time during the adaptive period is administered into an embryo and the cell chimaerism is deve

loping well Consequently it is possible to obtain such a high degree of heterologous tolerance between more closely related species by means of embryonic parabiosis, that it is most closely resembling the homologous tolerance. Thus a further series of interspecific parabiosis in turkeys, which had been parabionts with chickens revealed that chicken erythrocytes survived for a considerable period of time in two out of four individuals. In one case the graft transplanted in the second week after hatching survived in a good condition for three months and in the other case two skin grafts put on a one year-old animal survived as late as 80 days, when the animal died. In complete accord with the previously mentioned results it has been found that with the combination of turkey and chicken the immunization with foreign erythrocytes will induce a high degree of decrease almost a complete suppression of antibody formation. These results indicate that even a heterologous induction of tolerance has a chance of success, between zoologically closely related species at least.

THE SPECIFICITY OF IMMUNOLOGICAL TOLERANCE.

The acquired tolerance to foreign cells is strictly specific in intraspecific relationships. In a non inbred population skin homografts in tolerant animals take only when they are obtained from the original donor of cells. Though Cannon and others[8] found that in exceptional cases skin grafts from birds other than the donor of cells took well or were tolerated for a longer period of time. This can be explained on the basis of an accidental closeness of antigenic constitution of some individuals in a non inbred population However the very fact that homotransplantation tolerance is highly specific with respect to individual antigenic differences within the species remains unchanged

In accord with the concept that for the individual antigenic specificity of each individual a unique combination of a relatively limited number of antigens is responsible it was attempted to solve the possibility of obtaining tolerance which permits the acceptance of homografts from randomly chosen donors in the non inbred population Newly hatched ducklings are a specially suitable subject for this experiment because their immunological adaptive period for homografts lasts for several days after hatching It was found that all skin homografts in newly born ducklings take and induce tolerance (Hašková[22]). Survival of skin grafts transferred to ducklings of different age may be seen in Table IV

For this reason it was possible to administer a large amount of cells from a larger number of donors to newly hatched ducklings within several days after hatching Thus the newly hatched ducks were inject

TABLE IV — *Adaptive period for homografts in newly hatched ducks*

Grafting made after hatching in days.	Destruction of the graft within 30 days	Survives 80 days
1 — 5	0 %	100 %
7	50 "	25 %
21	100 "	0 "
28	100 %	0 "

ed within the first three days after hatching with approximately 2 10^8 of bone marrow cells through the intravenous route and approximately 1 ml of mashed spleen tissue through the intraperitoneal route. The cells needed for the inoculum were obtained from 60 newly born animals and every recipient was injected with a mixture of these cells. The recipients accepted well the injections of the given amount of cells from newborn animals and in the course of subsequent experiments no animal died or displayed signs of a « runt-syndrome » Morphologically different skin from leg which was transferred on the back of the animal was used as test homograft. Grafting was performed in at least three week-old animals and the results indicated that even in adult age, more than 50 % of skin homografts from animals selected at random took and exhibited a very high degree of tolerance (Hašek, Hašková [19])

The specificity in heterologous tolerance is rather a different question. In contrast with homograft tolerance the ability of individual specific recognizing of foreign antigens is not maintained. Grafts from any individual of the other species are tolerated. The decrease or suppression of antibody formation with respect to erythrocytes of various individuals of the other species is the same. Thus, in heterologous tole

TABLE V — *Production of immune agglutinins in ducks parabionts with chicks and control ducks, immunized with hen red cells (either from the partner in embryonic parabiosis or from another chicken)*

Donor (chickens)	Recipient (ducks) parabionts with chickens. Titre	Recipient (ducks) controls. Titre
Partner in embryonic parabiosis	2	32
	4	32
	8	64
		128
Control donors — —	4	32
	4	64
	4	64

rance the ability to respond to different antigens of individuals of the donor species is lost (Table V).

Finally it has been shown that in a turkey which had been a parabiont with a chick and tolerated skin heterografts *from chickens skin* homografts from another turkey were also tolerated. Two skin heterografts from two individuals and one skin homograft were placed at the same time on this turkey at one year of age. Also this graft survived for the whole time of observation—i.e. for 80 days, —while skin homografts in control turkeys were completely destroyed within 10-14 days. This turkey which had been a parabiont with a chick rejected another control guinea fowl heterograft *in the normal time The ability for* transplantation reaction against heterografts from other species was thus maintained The turkey was not a-gamma-globulinemic, gamma-globulin being demonstrated in the serum by means of paper electrophoresis. This case of a high degree of heterologous tolerance shows that in tolerance between turkey and chicken also the immunological response to individual antigens of birds own species is lost

CELL CHIMAERISM AND TOLERANCE INDUCED DURING EMBRYOGENESIS.

The question whether immunological tolerance can persist potentially i.e. *in absence of chimaerism is of special importance*

We have demonstrated in chicken parabionts that even one year after erythrocyte chimaerism had disappeared the inability to produce antibodies is maintained. Yet, the persistence of chimaerism of cells other than erythrocytes or persistence of antigens in the recipient's cells cannot be excluded

Also in complete heterologous tolerance interspecific chimaerism *is developing* By means of agglutination by a specific serum the presence of foreign erythrocytes can be demonstrated. In the majority of cases foreign erythrocytes in embryonal parabionts disappear within several weeks after hatching Yet, in accord with the observation of a complete heterologous tolerance we found turkey erythrocytes in a chick that had been in embryonic parabiosis with a turkey up to the age of 8 weeks (Hraba [11]) and in a guinea fowl which had been a parabiont with a chick the chicken erythrocytes could be demonstrated up to 3 months of age (Hort [12]). The longest interspecific erythrocyte chimaerism has been found in a previously mentioned turkey with a complete heterologous tolerance after more than one year Here chicken erythrocytes were demonstrable *up to its death at 14 months of age* It was also possible to demonstrate chicken erythrocytes in a blood sample withdrawn post mortem The majority of this bird's erythrocytes were chicken erythrocytes.

In this case of a long term interspecific chimaerism in addition to a serological evidence of the partner s erythrocytes we also attempted to demonstrate these foreign erythrocytes using the chimaera red blood cells as antigenic stimulus for immunization. The question arises of whether in this bird was not a « pseudochimaera » as observed in avian bird embryos following transplantation of adult foreign spleen cells from other species (Mitchison [34] Hort [24]) Transplantation of prospectively immunologically active cells from the chicken parabiont to the turkey partner might have occurred during embryonal parabiosis. These cells could probably acquire tolerance to the host s antigens. In postembryogenesis, however they might lose this heterologous tolerance and form antibodies against turkey agglutinogens, which would combine with the host s erythrocytes. Agglutination of these sensitized turkey erythrocytes by antiserum against chicken blood would thus stimulate the presence of chicken erythrocytes, i.e. it would be a case of pseudochimaerism. In order to exclude this possibility chicken agglutinogens were demonstrated in the blood of this chimaera by means of immunization. Chicken do not possess natural agglutinogens and do not form them even when immunized with turkey blood. Immunization of chicken with the blood of the chimaera however resulted in the formation of isoagglutinins which also agglutinated the erythrocytes of other chicken in a relatively high titre (Table VI). Individual chicken antigens were thus present in the blood of the chimaera, and induced formation of these antibodies. The demonstration of chicken antigens thus showed that this really was a case of a long term persisting of interspecific chimaerism.

In heterologous relationships we also encountered a case where erythrocyte chimaera has been not found and the heterograft was tolerated However also here the previously mentioned condition applies to that the chimaera of cells other than erythrocytes could persist. In the case of embryonal parabiosis the chimaerism must not always occur in post embryogenesis. One possibility is that hematopoietic cells were implanted in extraembryonic hematopoietic organs only and the chimaerism disappears together with the disappearance of this organ.

In any event, the dissociation of chimaerism from induced transplantation tolerance seems to be a difficult question. Erythrocyte chimaerism is developing not only after embryonal parabiosis but also following injections of foreign cells (Medawar [33]). The relationship between the chimaerism and the tolerance is of special interest in understanding the mechanism of immunological tolerance. There is no evidence hitherto that immunological tolerance could be maintained as a potential state without the persistence of antigen and in the case of transplantation tolerance without repopulation of the donor s cells.

TABLE VI

Plasma taken	*Chicken plasma No.*	*Erythrocytes of chicken No.*							*Titre of antiturkey hemagglutinins*
		728	734	882	883	885	886	7911	
Before immunization	2120	—/—	—/—	—/—	—/—	—/—	—/—	—/—	8
	2300	—/—	—/—	—/—	—/—	—/—	—/—	—/—	4
After immunization	2120	+/++	+/+++	+/++	+/+	+/+++	+/+++	+/++	128
	2300	—/—	—/—	—/—	—/—	—/—	—/—	—/—	64

Chick No 2120 immunized with blood of chimaeric turkey parabiont (age one year)
Chick No 2300 immunized with blood of control turkey
Citrated plasma used for agglutination reactions undiluted, if not stated otherwise Isoagglutination read off after 30 min./24 hours titre of anti-turkey hemagglutinins determined after 30 minutes
plasma diluted 1 10

TABLE VII — *Effect of intravenous injections of 2 10^7 cells from homologous or heterologous embryonic liver on survival of lethally irradiated mice*

Treatment	*Mice (No.)*	*Number of survivors days after exposure*				*Mean survival time*
		10	20	30	> 90	
None	110	11	0	0	0	7 7
Homologous embryonic liver	104	74	66	65	65	9,2
H terologous embryonic liver	52	0	0	0	0	5 0

Thus in solving the mechanism of embryonic adaptation to foreign antigens the same question arises as in the problem of the mechanism of antibody formation i.e. whether the presence of antigen is necessary or whether these mechanisms are capable of acting even in absence of the antigen

OTHER TYPES OF INDUCED TRANSPLANTATION TOLERANCE.

Among other well known models of experimentally suppressed immunological reactivity the immunological paralysis, as described by Felton [11] appears to be an immunological phenomenon closely related to acquired tolerance in embryos and in very young animals. Yet, it differs in that it is possible to produce it in immunologically mature animals, whereas the orthodox transplantation tolerance may be elicited in immunologically immature organisms during the so-called adaptive period only Many efforts trying to produce transplantation « paralysis » in the adult age brought so far essentially negative results (Castermans [9] Zotikov personal communication) The question remains, however to what extent the difference between the two phenomena is substantial.

A further promising approach to obtaining transplantation tolerance especially in adult animals, is irradiation of the recipient with a total body lethal dose of X rays. The lethally irradiated animals may be induced to recover by a subsequent injection of homologous and sometimes heterologous cells from bone marrow spleen and certain embryonic tissues. The transplanted skin homo- or heterografts on these animals survive even when their normal immunological reactivity has been recovered This type of induced transplantation tolerance however differs from the former in the mechanism of its occurrence The first type of transplantation tolerance is the outcome of a specific adaptation of the immature lymphoid cells in embryos, whereas after irradiation tolerance develops as the outcome of the elimination or the permanent inhibition of the adult recipient's own mature lymphoid cells. This remarkable phenomenon was gradually explained by the works of various authors (Jacobson [24] Lorenz et al [26] Main and Prehn [32] Lindsley et al [29] Loutit et al. [31] Šoška et al [37])

Successful tissue grafts on irradiated and recovered organisms are actually autografts with respect to immunologically active cells of the recipient

In our laboratory transplantation tolerance in lethally irradiated animals was studied by A. Lengerová [27, 28] In lethally irradiated recipients of non-irradiated hematopoietic tissues different conditions for immunological reaction between the host and the graft may occur on

the basis of the genetic relationships existing between the recipient and the donor Such a model type of experiment is represented by C57BL inbred mice using different sex combinations of the recipient and donor In this strain the transplanted male tissues are very uniformly rejected by female hosts but not conversely (Eichwald et al.[14]). The results of our *experiments undertaken on 50 mice* C57BL irradiated with 900 r X rays are as follows

1 Isologous male bone marrow caused 100 % survival in irradiated males for more than 90 days after exposure.

2 A part of (3 of 10) female mice treated with isologous male bone marrow died during the second and third week after exposure but no cases of death occurred later

3. All males treated with isologous female bone marrow survived the initial 3 weeks period most of them (8 of 10) however died during the next seven weeks.

4. Males treated with homologous male bone marrow died either in the initial period after exposure (4 of 10) or in the period of delayed death (6 of 10).

5 All nontreated controls died during 11 days after exposure

These experiments showed, therefore that during the first three weeks after exposure death occurred only in these groups where conditions were fulfilled for the immunological reaction of the host against the donor cells on the contrary the delayed death took place only in the groups where genetic conditions for the « graft versus host » reaction were fulfilled

It would seem therefore that immunological reaction in both directions *between irradiated recipient and foreign* cells *graft* could play a role in the smaller therapeutic effectiveness of the homologous hematopoietic cells in comparison with that afforded to irradiated animal by isologous tissues.

We supposed that if the graft versus host reaction took place in radiation chimaeras, it *could* be prevented using homologous or heterologous hematopoietic cells from embryonic donors provided that immunological tolerance could be induced in a graft of embryonic lymphoid tissue to its tolerant adult host Experiments of Lengerová[11] showed this possibility to be real for in the homologous combination of the adult irradiated recipients and embryonic donors of hematopoietic cells the delayed death did not occur

In further *experiments we compared* the effectiveness of homologous and heterologous embryonic liver cells to induce recovery of lethally irradiated mice. The mean number of nucleated cells injected per mouse was $2 \cdot 10^7$ Summarized results from several experiments are presented in the Table VII

The injections of rat embryonic liver cells did not exhibit any positive effects, on the contrary they were apparently harmful for the irradiated recipients that died sooner than nontreated controls. This fact is surprising regarding the at least transitory beneficial effect of hematopoietic cells from adult rats. Experiments are in progress to explain this discrepancy

The homologous embryonic liver cells induced 62 % of the otherwise lethally irradiated mice to recover no cases of delayed death occurred among the survivors of the initial 21 days period

This finding seems to be in agreement with the hypothesis that immunological tolerance to the host can be induced in the embryonic graft. An alternative hypothesis could be considered that the embryonic lymphoid elements do not mature in the irradiated host at all and therefore their tolerance to the host is a nonspecific one. This possibility requires further investigations.

Thus both types of transplantation tolerance induced either by embryonic adaptation or by massive irradiation represent two hitherto most effective experimental models towards overcoming tissue incompatibility under experimental conditions. In both cases the number of theoretical problems which research on these phenomena raises is still considerably greater however than those for which answers have already been found. Finally the possibility cannot be excluded that induced transplantation tolerance could find a practical application even in the clinical field.

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DISCUSSION

MITCHISON I should like to ask Dr Hašek if he has made any attempts to modify the state of tolerance in his heterologous system by passive antibody

HAŠEK I am sorry but we have not tried

WOODRUFF I have wondered for a little time whether we are not assuming what we are setting out to prove when we speak of tolerance as being quite so completely specific When results turn out that do not fit in with this, we tend to talk of overlap of antigens and we say that the antigens that do not overlap are weak ones and do not very much matter It seems to me that Makinodan and his colleagues at Oak Ridge have shown pretty clearly that the radiation chimera is an immunological cripple and we ought perhaps to look a good deal more critically than we have done so far at the general immunological status of animals which have been made chimeras by injection in the neonatal period

OWEN Dr Hašek you have made the point that animals rendered tolerant by parabiosis retain their specificity of reaction to other anti

gens. Do you have any quantitative data as to whether they produce as much antibody or as high titred an antiserum as the normal non-para biotic bird would ?

HAŠEK In answer to Dr Woodruff's comment, I agree that in non inbred populations one can have tolerance which is not strictly specific to be individual but this does not disprove the specificity of tolerance in respect of particular transplantation antigens.

As to Dr Owen's question We have made quantitative compari sons but only of course, in cases of heterologous tolerance by estimating the titres of agglutinins.

WOODRUFF May I just elaborate very briefly this point ? It is claimed, as Medawar said earlier that the transplantation antigens of skin thyroid, adrenal and other tissues are common, and the criterion we use to show this is a tolerance test. Now if in fact the animal in which we are doing the test is immunologically a little under-privileged then we may very well be drawing erroneous conclusions and I believe we have got to get at this by a much larger scale study of the reactions of these creatures to a variety of antigens.

MEDAWAR What Woodruff says might I think apply to experiments in which tolerance is induced by the injection of adult spleen cells but it does not apply to Dr Hašek's experiments because, in many of these, tolerance was produced by embryonic parabiosis, so that there could be no runt disease complications to produce the immunological crippling which Woodruff has referred to

OWEN The work of Makinodan and his colleagues to which you referred Dr Woodruff is subject to the same kind of reservation We dealt with heavily irradiated animals which had good reason to be im munological cripples

BRENT May I add a comment on this question of individual or strain specificity ? Billingham and I have been studying strain-speci ficity in tolerant mice, and our results indicate that a mouse made tole rant by neonatal injection of homologous spleen cells, is perfectly capable of reacting against skin grafts from a third strain provided that this third strain is genetically dissimilar to the donor strain with whose tissues the mouse was made tolerant. To give an example if an A strain mouse is made tolerant with C3H cells and tested with CBA skin grafts, the tolerance will be found to extend to some extent to the CBA

grafts. This merely indicates that there is a considerable degree of genetic affinity between C3H and CBA strains, and this is of course known from other data. The same applies the other way round. If an A strain mouse is made tolerant with CBA cells it will display a partial tolerance towards C3H skin grafts. If on the other hand, a C3H mouse is made tolerant with CBA cells and tested with A skin homografts, the A skin homografts will be rejected as quickly as they would have been destroyed by a C3H mouse which had never been made tolerant at all. This finding indicates that the CBA and A strains differ by many antigens. So when we talk about strain or individual specificity of tolerance we really mean that the animal can clearly distinguish between those antigens of which it was made tolerant and others which were *not represented in the tolerance inducing inoculum*. But due to the antigenic overlap which is sometimes found between individuals or mouse strains tolerance may at times extend to third parties.

VOISIN: I would like to ask Dr Brent what is the median survival time of grafts made from C3H to CBA or CBA to C3H?

BRENT: On normal animals?

VOISIN: Yes.

BRENT: The degree of genetic similarity or dissimilarity between two strains is of course reflected in the survival time of grafts on normal adult animals, and in this case the survival time is about 15 or 16 days; this is much longer than the survival time of say a CBA graft transplanted to an A strain mouse.

VOISIN: It fits perfectly well.

OWEN: What are the effects of pre-immunization between these two strains; if one injects CBA cells into C3H and then does a skin graft, does one observe a second-set response?

BRENT: We have *not* studied pre-immunisation in this particular strain combination. With two other strains we have found that immunisation of A strain mice with CBA grafts resulted in a pretty strong immunity against grafts from our AU strain, indicating a sharing of antigen between the CBA and AU strains.

OWEN: Experiments along this line might bear on the question raised by *Dr Woodruff*. It seems to me from the literature that second

set responses, when the second graft is from a different strain than that used for the primary graft occur less frequently than one would expect. One would expect rarely to find a combination in which there were not some antigens similar or shared between the primary and the secondary sensitizing test strains. On the other hand, in the tolerant situation it seems that one frequently postulates similar or shared specificities to explain cross tolerance. So I just wonder whether there may not actually be some reason for believing that tolerance is less specific in this sense than immunisation is.

BRENT I am not sure that this question which you raise, and which is clearly a very important one has been thoroughly studied in the mouse.

MITCHISON I think I can add something to this discussion from chicken material, not from mouse material. I think one's theoretical expectation here is that in a situation in which tolerance is fully specific you might get cross-reactions in the immunisation system, and what you are saying is that this is not the case in the mouse and this is puzzling Now in the chicken I think that this is so and I can give one example of this if you use Brown Leghorns as recipients then White Leghorns will immunise against Light Sussex and vice versa but in the tolerance situation (using tolerance to erythrocytes) then a Brown Leghorn which is tolerant of a White Leghorn will still show full immunity to a Light Sussex

Skin transplantation immunity in relation to hypersensitivity reactions of the delayed type

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INTRODUCTION

This paper is intended to be a contribution to our very meagre knowledge of how the homograft reaction takes effect.

The evidence recently summarized by P.A. Gorer [7] makes it almost obligatory to abandon a « unitarian » theory of the homograft reaction, i.e. the theory that all homografts of all tissues are destroyed by essentially similar mechanisms. Gorer distinguishes between reactions of three general kinds: those which are directed against (a) leukotic cells, (b) ascites tumour cells proliferating freely in the abdominal cavity, and (c) « solid » homografts like orthotopic skin homografts which establish primary vascular and lymphatic connections with their hosts. The work discussed in this paper bears upon the third of these three reactions and may have no direct bearing on the other two.

For the sake of argument we may adopt one or other of two extreme views about the immunological process that is responsible for the destruction of orthotopic skin homografts: (a) that it takes effect through the action of orthodox serum antibodies, and is therefore analogous to most anti-bacterial and anti-viral immunities, the reactions elicited by soluble antigens, the iso-immune reactions that reveal themselves as blood-group incompatibilities, the Arthus reaction and cutaneous sensitivity reactions of the « immediate » type; or (b) that it takes effect through the topical action of activated lymphoid cells which behave as if they were transporters of a cell-bound antibody (cp. Billingham and Brent [1]). To this second class belong the immunological reactions which can be made to manifest themselves in guinea pigs and

human beings as hypersensitivities of the « delayed » type it includes therefore, the drug and bacterial allergies the reactions that can be elicited by the injection of antigen antibody complexes (Uhr Salvin and Pappenheimer[19]) and possibly that component of auto-immune reactions which is responsible for the destruction of tissues.

The evidence for and against the two extreme views has been the subject of several recent reviews (Gorer[7] Medawar[13] Brent[5] Voisin[17]) In bare outline it is as follows (*) In favour of the purely humoral hypothesis is firstly the fact that homografts of normal cells and tissues (not excluding skin) do indeed elicit the formation of a variety of serum antibodies haemagglutinins haemolysins (Hildemann[9]) leuko-cyte agglutinins « protective antibodies » and cytotoxins Secondl[y] the antigens that cause the formation of these serum antibodies ar[e] widely distributed in the fixed tissues the use of red cells to disclos[e] their presence is purely a matter of technical convenience. Thirdly these antigens have a common genetic determination with antigens that are known to give rise to transplantation immunity (This third proposition has been shown to be true only of mice, but there is no definite evidence against its being true of other animals as well) These three sets of facts make a strong *prima facie* case in favour of a humoral hypothesis—which is by no means to imply that the humoral anti body which may be responsible for the destruction of skin homografts belongs to any of the categories mentioned above.

The evidence in favour of the cellular hypothesis is partly direct and partly circumstantial much of it is evidence against the particip-ation of serum antibodies rather than evidence which demands the acceptance of a cellular hypothesis in its own right. The most impor tant single piece of evidence, which Mitchison was the first to provide, is that transplantation immunity can be transferred from one animal to another by means of activated lymphoid cells but not, so far as present evidence goes by immune serum even when administered in heroic doses. On the contrary an immune serum containing demonstr ably high titres of agglutinating antibodies more usually *weakens* the homograft reaction the « enhancement » which it can be made to procure has indeed been formally defined (Kaliss[10]) as a prolongation of the life of tumour homografts brought about by their exposure to the action of specific antibodies possibly haemagglutinins. The work of Hildemann[9] on goldfish adds further weight to the now gener accepted belief that there is no correlation between the presence absence of haemagglutinins and the presence or absence of transpla tation immunity

(*) Citations of evidence will be confined to the more recent literature

Skin transplantation immunity in relation to hypersensitivity reactions of the delayed type

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INTRODUCTION

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human beings as hypersensitivities of the « delayed » type: it includes, therefore, the drug and bacterial allergies, the reactions that can be elicited by the injection of antigen-antibody complexes (Uhr, Salvin and Pappenheimer [19]) and possibly that component of auto-immune reactions which is responsible for the destruction of tissues.

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The question of the complicity of humoral antibodies in skin transplantation immunity has been raised anew by the work of Stetson and Demopoulos[11] who report that serum from mice and rabbits which have been sensitized by lymphoid cells combined with Freund's complete adjuvant can transfer transplantation immunity to normal subjects i.e. can cause them to behave towards skin homografts as if they themselves had been sensitized by living homografts beforehand. We are engaged in a full repetition of Stetson's work so far we have not been able to reproduce his findings, but we cannot come to a final conclusion until certain variations in the execution of the experiments have been explored (*)

There is, however much other evidence against the participation of humoral antibodies in the homograft reaction considered individually no one piece of evidence is decisive, but together they are of some weight. The ingenious work of the late G.H. Algire and his colleagues on the *behaviour of homografts enclosed within membranes of various* degrees of porosity shows either that humoral antibodies are inactive or that they act only at the high concentrations that prevail in the immediate neighbourhood of the cells that make them. Foetal sheep can react upon skin homografts at an age at which they are thought to be incapable of manufacturing antibody globulins. Several energetic attempts to demonstrate a cytotoxic action of immune sera on epithelial cells *in vitro* have failed (but in Medawar's experiments sensitized regional lymph node cells were also inactive *in vitro*). Finally the homograft reaction in guinea pigs can be made to express itself as a cutaneous hypersensitivity of the delayed type, a property which forms the subject of the new experiments described below

The *property that unites the so-called « delayed hypersensitivity reactions » is not merely the symptomatic expression which gives them* their name above all it is the fact that *they are transferable by activ*ated lymphoid cells and not by serum. We shall now describe experiments upon guinea pigs which show (a) that an impure antigen which is known to be capable of eliciting transplantation immunity is also capable of eliciting a tuberculin like reaction when injected intradermally into animals which have been sensitized by skin homografts and (b) that this tuberculin-like reactivity can be transferred from one animal to another by means of activated lymphoid cells but not by means of « immune » serum We shall consider these two phenomena separately under the headings of the *direct* reaction and the *transfer* reaction.

(*) See Appendix

METHODS

Our techniques have been described in adequate detail in a previous report (Brent, Brown and Medawar [4]) and the following summary is merely intended to provide a background for general discussion.

In their simplest form, both the direct and the transfer reactions make use of a pair of guinea pigs, viz. a recipient R which has been sensitized by skin homografts from a donor D. The direct reaction is evoked by the intradermal injection into R of « antigen » taken from D: the antigen we have used has been *either* a cell-free extract made from the spleen of D by the method of Billingham, Brent and Medawar [3] *or* a suspension of living cells from the lymph nodes of D. The transfer reaction is evoked by the intradermal injection into D of cells from the regional lymph nodes (the retroscapularis nodes) of R. To avoid confusion it must be emphasized that the living lymphoid cells which elicit the direct reaction are used purely as a convenient source of antigen: they are easy to prepare virtually free from red cells, and can be obtained without killing the donor. In the transfer reaction, however, they represent a source of immunologically activated cells, i.e. of cells which have acquired a specific immunological reactivity as a result of their exposure to antigenic stimuli from D homografts.

Both the direct and the transfer reactions are « tuberculin-like ». They have been scored by reference to an arbitrary scale ranging from + to + + + + + and based partly upon the area of inflammation and partly upon its intensity in terms of colour and degree of swelling. The injection of *normal* lymphoid cells into *normal* guinea pigs in dosages of 30m ($m = 10^6$) downwards has not, in our experience, elicited a reaction greater than +: except where it marks the end-point of a series of dosage dilutions we do not regard a score of less than + + as definite evidence of a positive response.

When both are elicited in a single pair (D and R) of guinea pigs the direct and transfer reactions are strongly correlated in their degree of intensity—as would be expected if, as we suppose, both depend upon the interaction of D antigen with sensitized cells from R.

THE DIRECT REACTION

The direct reaction has a latent period of four to eight hours and reaches its peak between the 24th and 36th hour. Its intensity varies (a) with the degree of sensitivity of the recipient as shown (not always reliably: see below) by the survival times of the homografts used to sensitize the recipient R and (b) with the dosage of antigen. Living

donor cells which have provoked a + + + + + reaction at a dosage of 12m still elicit a clearly positive response at a dosage of 2m and when the cell free antigen extracted from 50mg initial wet weight of spleen has elicited a + + + + + reaction, a reaction is still perceptible after the injection of one tenth of this quantity The injection of antigen of *isologous* origin into sensitized recipients has evoked no response. Evidently then the existence of a state of sensitivity as such is not sufficient to account for the direct reaction the sensitivity *must* be specifically directed against some at least of the antigens present in the inoculum used in the skin test

It may be a matter of some theoretical importance that R guinea pigs which in addition to D skin homografts, had received repeated weekly *injections* of D *whole blood*, did not become more reactive in the skin test some, indeed, became less reactive. Yet when challenged anew with D skin homografts at the end of the course of injections these « hyper immunized » animals destroyed them at the rate characteristic of a second-stage response.

It is not obligatory to sensitize animal R with *skin* homografts from D but no other method of sensitization provides a visible outward record of the immune status of the experimental subjects—a matter of great importance when using outbred animals of unknown genetical composition.

In an attempt to find out how quickly an animal becomes demonstrably sensitive after exposure to foreign homologous cells we have injected donor lymphoid cells by the intravascular route into R guinea pigs in dosages ranging from 21 to 160m The earliest clearly positive direct reaction (+ + or greater) was secured when the recipients were challenged with antigen three days after the injection In guinea pigs which have been sensitized by homografts of skin the direct reaction remains clearly positive for at least a month after the homografts have broken down

Extracted splenic antigen gives more regular results in the direct reaction than living donor cells and is to be preferred on all counts except speed and convenience of preparation.

The transfer reaction.

The intradermal injection into the donor D of 5m to 10m cells from the lymph nodes of the sensitized recipient R produces an inflammatory reaction with a latent period of four to eight hours and a peak between the 36th and 48th hour after which it fades slowly away The transfer reaction therefore takes longer to reach its maxi

mum than the direct reaction and declines more slowly. The inflammation that accompanies it is usually more intense.

The earliest time after the transplantation of the sensitizing skin homografts at which the regional lymph nodes become just perceptibly competent to elicit the transfer reaction is, in our experience, between four and six days, but their ability to do so is known to persist for at least 40 days i.e. for at least a month after the sensitizing homografts have broken down. A comparison of the regional, contra lateral, cervical and mesenteric nodes shows that competence to elicit the transfer reaction is not confined to cells taken from the regional nodes but that the regional nodes are usually more active, and never less active, than the remainder. The mesenteric nodes have been active in only two out of five trials. Testicular cells and cells from the submaxillary salivary gland (a particularly convenient and accessible source) elicit no reaction.

The injection of demonstrably activated lymphoid cells into isologous recipients—i.e. into animals isogenic with those from which the sensitized cells are taken—does not elicit a transfer reaction. Evidently the reaction depends not merely upon the « sensitization » of the transferred cells: it depends upon a sensitization directed towards antigens present in the animal whose tissues provoked the state of sensitivity in the first instance. (The presence of these antigens in the donor D does not, of course, imply that they are absent from any other guinea pig chosen at random from a heterogeneous stock: it is for this reason that all experiments other than those making use of inbred guinea pigs have been based upon a strict pairing of donors with recipients.)

We have not so far been able to elicit the transfer reaction with cells killed by heating to 48.5 C, by drying in the frozen state (« lyophilization ») or by freezing and thawing. Nor have we been able to elicit the transfer reaction with *serum* taken from animals whose lymphoid cells are demonstrably active, or which, in addition to skin homografts, have received repeated weekly injections of donor blood. Both « immune » serum and normal homologous serum sometimes produce a weak and diffuse inflammation around the site of injection which may persist for 24 hours.

Homografts exchanged at random between guinea pigs of unknown genetic origin sometimes survive longer than the modal average period of eight to twelve days. This has made it possible for us to execute a useful type of control experiment in which the transfer reaction is attempted with regional lymph node cells from animals whose homografts are still intact. These reactions have invariably been negative nor do they rise above a low level of intensity (+ +) even when these unusually long lived homografts have eventually broken down. On the

other hand the transfer reaction has not always been strongly positive even when the sensitizing homografts have broken down within 12 days. The cause of these relative failures is not yet understood. *It is conceivable* that the transfer reaction depends for its success upon the participation of « strong » histocompatibility genes analogous to those determined by the H 2 locus in mice. The survival of skin homografts beyond 12 days implies, almost by definition, that antigens of this strength are not implicated but survival *for less than 12 days does not by any* means imply that they are. A comparable situation exists in mice: the survival times of skin homografts transplanted from A to CBA mice and *vice versa* are much the same, but only the combination A-to-CBA involves incompatibility with respect to a « strong » H 2 allele.

Discussion.

1. *The homograft reaction as a cellular immunity*

According to our present interpretation both the direct and the transfer reactions are symptoms of a local interaction between donor antigen and the recipient's sensitized cells. In the direct reaction cell-free antigen (or living donor lymphoid cells acting as a source of antigen) is injected into the skin of a sensitized recipient. The cells with which *it thereupon interacts are presumably lymphoid* cells captured from the systemic circulation (*). In the transfer reaction sensitized lymphoid cells are injected into the skin of the homograft donor, where antigen can be assumed to be abundant. Except that the antigen which participates in it is ready-made, the transfer reaction is thus in the usual terminology a « reversed passive » reaction—passive because the primary immune response is undertaken by an animal other than the subject of the experiment, and reversed because sensitized cells introduced locally confront antigen which has a systemic distribution.

Taken at their face value our experiments establish a clear affinity between the homograft reaction and the tuberculin and drug allergies. In the first place the reaction can be made to express itself as a cutaneous inflammation of delayed *onset in an animal which is notoriously* susceptible to bacterial and drug allergy, and the inflammatory response is broadly similar to a tuberculin reaction both histologically and in outward appearance. (Our attempts to elicit either direct or transfer reactions in normal or adrenalectomized mice, whether in quiescent or actively hair-growing skin, *have been completely* unsuccessful). In the

(*) An alternative but less plausible possibility is that cells belonging to resident population of the skin itself (e.g. vascular endothelial cells) have somehow been made sensitive to donor antigen.

second place the reaction can be transferred by living sensitized lymphoid cells, but not by cells which have been killed by heating to 48.5° C, by repeated freezing and thawing, or by drying in the frozen state. Nor has it yet been transferred by « immune » serum.

We do not suggest that the analogy is so exact as to amount to a complete formal similarity The tuberculin reaction is transferable by blood leucocytes and the cells of sterile inflammatory exudates Technical difficulties have so far prevented our testing thoracic duct lymphocytes or blood leucocytes for their ability to elicit the transfer reaction in guinea pigs, but peritoneal exudate cells have been used with great success. Although it would be premature to arrive at a final conclusion we can say that neither the direct nor the transfer reaction are totally abrogated by cortisone or antihistamine. The homograft reaction in mice, rabbits guinea pigs and (Billingham and Hildemann [4]) hamsters is cortisone sensitive in so far as primary immunization is delayed by the administration of cortisone but, except under very special circumstances (Krohn [11]) the second-set reaction given by an animal which has already been immunized by homografts is unaffected Again the antigen (OT or PPD) which in the tuberculin test is used to reveal the existence of a state of sensitivity is not the same as that which provokes it in the first instance At present the antigen which we use to elicit the direct reaction is « complete » but future work may show that something less than the complete antigen will suffice. Until these various uncertainties have been resolved it would be wisest to classify the homograft reaction as a reaction *sui generis*, though it obviously resembles a cellular immunity much more closely than an immunity of the familiar humoral type.

2. *The effector mechanism of the homograft reaction*

The reaction against « solid » homografts can be classified as a cellular immunity for two reasons (a) because humoral antibodies in the quantities and concentrations at which they occur in the serum of demonstrably sensitive animals are not sufficient to account for their destruction and (b) because the destruction of solid homografts seems to depend upon the intercession of activated lymphoid cells These two statements are sometimes taken to imply that humoral (or orthodox soluble) antibodies play no necessary part in the cellular immunities but this, though it may be true, is a *non sequitur* The activated lymphoid cells that transfer sensitivity to homografts can also transfer orthodox humoral iso-immunity (Mitchison and Dube [14]) One possibility then is that they produce effective humoral antibodies but in quantities so small that their action can only be detected in the

immediate neighbourhood of the cells that make or carry them. As Gorer[7] has pointed out such an interpretation could accommodate the observations of Algire and his colleagues on the behaviour of homografts enclosed within membranes that are permeable to blood solutes but not to cells. It might also explain the fact that the regional lymph nodes of mice sensitized by homografts can inactivate a cell free « homograft antigen » (Berman and Brent[1]) (The possibility that hyper immune serum can inactivate antigen is under investigation now) If this interpretation is true our complete failure to transfer immunity by the local intradermal injection of dead cells is a little surprising It can be argued however that the concentration of anti bodies within sensitive cells at any one time is so small that the transfer reaction must depend upon their continued manufacture, and therefore on the metabolic integrity of the transferred cell. The improved methods of extraction which we are now attempting should however be able to reveal the presence of soluble antibodies in sensitized lymph oid cells if they are in fact there.

A second possibility is that the « antibodies » through which the cellular immunities are mediated are structural rather than soluble, i.e. that they are « endo-antibodies » bearing the same relationship to free serum antibodies as the enzymes of the cyclophorase system bear to the glycolytic enzymes or to enzymes which are secreted into the sur rounding medium Further analysis of the transfer reaction should eventually enable us to distinguish between these two interpretations. In terms of the number of cells needed to reveal it (5m to 10m) it is much more sensitive than other transfer reactions that depend upon the use of cells. Above all the fact that the transferred cells or extracts made from them are injected *locally* should go far to avoid the objection that takes away much of the significance of attempts to transfer the cellular immunities with sub-cellular material viz that sub-cellular matter never reaches the place where it was intended to go, or is inactiv ated before it gets there. In the transfer reaction as we have described it we believe that we can attach special weight to negative results

The fact that the activated lymphoid cells which are used to transfer transplantation immunity can also transfer orthodox humoral im munity cannot be construed as evidence that cells of the same type are responsible for both cells expressed from lymph nodes are a very heterogeneous population Although it is possible that the « sensitiz ation » of lymphoid cells is simply an episode in a process which ultimately leads to the liberation of orthodox exo-antibodies it is equally possible that the cellular and humoral immunities employ pathways of response that distinguish them from the outset We urgently need to know what part is played in the homograft reaction by cells present in

the leucocyte population of peripheral blood. The work of Simonsen[14] and of Billingham and Brent[2] on the production of runt disease or allied phenomena by leucocytes shows that blood does indeed contain an immunologically competent cell. It is conceivable that this cell is the prime mover in all immunological reactions that are initiated by sessile antigens.

Even if we were able to answer all the questions put above—even if, for example, we were entitled to infer that the cells which intercede in the homograft reaction are quite distinct from those which are responsible for humoral immunity and contain no antibodies other than structural endo-antibodies—we should still not know what exactly it is that kills a homograft. The interaction between antigen and antibody may itself be lethal, or it may be mediated through a non-specific factor playing the same part as complement plays in haemolysis. Humoral antibodies of some kind may be necessary for the reaction even if they are not sufficient. Again, homografts may be destroyed by an inflammatory process which is specific only in the sense that it is an immediate secondary consequence of a specific union of antigen and antibody. We do not even *know* what it is that causes primed or activated lymphoid cells to congregate in the neighbourhood of their target. The mere circulation of activated cells can be of no avail unless they can make use of some immunologically specific process of recognition. One possibility, which we are now investigating, is that sensitized lymphoid cells are destroyed by contact with specific antigen, in a manner analogous to the action of tuberculin on tuberculin-sensitive cells (Rich's phenomenon). A second possibility (cp. Berrian and Brent[1]) is that, if antibodies are present on their surfaces, activated cells may be arrested in the neighbourhood of the homograft.

SUMMARY

A tuberculin-like reaction is produced by the intradermal injection of cell-free antigenic matter into guinea pigs which have been sensitized by skin homografts from the antigen donor. This reaction can be transferred by the intradermal injection into the homograft donor of living lymphoid cells from the sensitized recipient. It has not been transferred by dead lymphoid cells, living non-lymphoid cells, or immune serum.

These results add weight to the hypothesis that the reaction against skin homografts is mediated through cells rather than through serum antibodies.

The activated lymphoid cells which give effect to the homograft reaction may produce some kind of orthodox soluble antibody in quantities so small that its effects are discernible only in the immediate neighbourhood of the cells which make it. Alternatively, the antibodies may be insoluble or structural ("endo-antibodies"). Transplantation immunity may be mediated through the same cells that produce humoral antibodies or it may employ a pathway of response that is distinct from the outset.

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APPENDIX

ATTEMPT TO TRANSFER IMMUNITY AGAINST MOUSE SKIN HOMOGRAFTS WITH SERUM FROM ADJUVANT SENSITISED DONORS. (See p 118 of text)

The experiments briefly summarised here represent an attempt to confirm in the mouse the work of Stetson and Demopoulos[15] whose experimental design we have followed in every respect but one (see below). Mice of one inbred strain (« serum donors ») were sensitised with a suspension of spleen cells from mice of another strain (« spleen donors ») the cells having first been emulsified with human tubercle bacilli suspended in Freund's incomplete bacto-adjuvant (Difco). The cell-adjuvant mixture was injected into the four foot pads as well as subcutaneously Ten to 20 days later the serum donors were bled by cardiac puncture and the pooled sera injected intravenously into groups of mice belonging to the same strain. In the majority of experiments the serum dose was 0.5 ml but one group of 13 mice received only 0.1 ml Skin grafts prepared from animals isologous with the spleen donors were transplanted to the chests of all serum recipients within 1 hour of serum transfer The technique of skin grafting used by us has been described by Billingham and Medawar[21] and more recently by Brent[12] and the method used for the appraisal of the fate of the test-grafts has been fully discussed elsewhere (Billingham, Brent Medawar and Sparrow[24] Billingham Brent and Medawar[19]). In brief the plaster bandages were removed on the 6th post-operative day and the grafts biopsied after careful inspection histological study revealed the precise degree of survival of the grafts epithelial elements as well as other features such as the

degree of vascularisation In the strain combinations used by us 6-day homografts on non sensitised mice invariably have a fully or very nearly fully surviving epithelium. However previous sensitisation of the graft recipients with skin grafts spleen cells or non-cellular extracts results in the total or partial destruction of the grafts epithelial elements by the 6th day the extent of destruction depending upon the strength and timing of the sensitising stimulus. It should therefore be noted that the main criterion for immunity has in our experiments, been based upon the curtailment of survival of skin homografts rather than on the degree of vascularisation alone (though the latter has also been taken into account)

Some of our experiments were conducted with mouse strains (Strong A and CBA) differing from those used by Stetson and Demopoulos (A/Jax and Balb/c) in others, however we have used Strong A serum donors and Balb/c spleen donors

Besides comparing the fate of grafts on immune serum recipients and normal animals, a further and perhaps more discriminating control was introduced In several experiments groups of mice were injected with sera prepared from mice previously treated with *isologous* spleen cells in adjuvant, the experimental procedure otherwise being strictly comparable with that used for homologous sensitisation

The results are summarised in the Table. It will be seen that, using the criterion of graft survival immune serum had little or no effect. That the somewhat low scores of some of the grafts in the first two experiments cannot be regarded as having been caused by a specific phenomenon is indicated by the finding that a similar effect was mediated by serum obtained from mice injected with isologous cells. We are at present at a loss to understand why isologous cells should be capable of producing such an effect, for although experimental procedures of this kind have been used to elicit auto-immune responses it seems unlikely that auto-antibodies can have played a role in the context of these experiments (*) Although there is at present no experimental evidence in its favour it is not impossible that the presence of the adjuvant itself elicited an immunity which on transfer interfered with the vascularisation of the grafts.

Some of the mice sensitised with homologous cells in adjuvant were themselves grafted with skin from the spleen donor strain in order to ascertain that the sensitising procedure had been successful. Column 6 of the Table shows that, with the exception of the last two experiments, the active immunity in the serum donors was far from maximal In the last two experiments (Balb/c to A) the 30 potential serum donors

(*) But see Voisin, Toullet & Maurer (2)

Attempts to transfer immunity against mouse skin homografts with serum from adjuvant immunised donors

Strains	Interval (days)	Serum dose (ml)	Homolog immunis * (% survival)	Isolog. immunis (% survival)	Active immunis. (% survival)	Straight controls (% survival)
CBA → A	12	0.5	100, 75 75, 50, 50 25	100, 90 90 80 75	—	—
CBA → A	15	0.5	100, 100, 100 90, 75, 50, 50, 50	90 75, 75, 50, 50	25 25 10 10 5, 5, 5 0	8 × 100
A → CBA	20	0.5	6 × full survival	5 × full survival	—	8 × 100
Balb c → A	15	0.5	100, 100, 100, 100, 100, 100, 90	100, 100, 100 100 75 50 50	75, 25 25, 10 10 0	8 × 100 3 × 90 1 × 50
Balb c → A	12 + 6	0.5	11 × 100 90 90, 75	—	26 × 0 4 × full survival	—
Balb c → A	12 + 6	0.1	10 × 100 75 25 25	—		

Interval between sensitization and serum transfer
Serum donors injected with homologous cells in adjuvant
Serum donors injected with isologous cells in adjuvant
Skin grafts transplanted to sensitized serum donors
Skin grafts transplanted to non-sensitized mice
No biopsy grafts normal and without deviation from expected life span

See text.

were therefore grafted with homologous skin 12 days after sensitisation thus permitting the selection of only strongly sensitised mice for serum transfer. It so happened that as many as 26 of these mice proved to be strongly immune, and their sera were therefore used in experiments involving a total of 27 recipients. Again no convincing evidence of a passive transfer of immunity was obtained. On the contrary the great majority of the grafts displayed exuberantly hyperplastic epithelia and entirely normal vascularisation. This was particularly true of test-grafts which had received the high dose (0.5 ml) of serum and suggests the possibility that the serum was, if anything, protecting the grafts against the incipient response of their hosts (« enhancement »). Of the grafts transplanted to recipients of low serum doses (0.1 ml) two—marked in the Table—exhibited extensive epithelial autolysis and a total failure to establish vascular connections. We cannot be certain that the condition of these isolated grafts was brought about by a passive immunity or by non-specific factors such as faulty surgery. Curiously enough two grafts very similar in appearance were found in a group of 6 mice injected with 0.25 ml of serum obtained from donors which had evidently not been successfully sensitised by the homologous cell-adjuvant mixture as indicated by the survival of their own test-grafts 6 days after transplantation. Furthermore, total avascularity with attendant epithelial destruction was also observed in a portion of a graft removed from an animal in the isologous control group of a previous experiment. We are therefore inclined to believe that the condition of the two grafts in the experimental group was caused by non-specific factors. Nevertheless in future experiments of this kind it might be advisable to use low rather than high serum doses.

Finally we wish to record that the repeated injection of anti-CBA serum (prepared as described above) into tolerant A strain mice carrying CBA skin grafts did not in the least prejudice the survival of the well-established grafts despite the fact that the serum was transferred intravenously, intraperitoneally and subcutaneously (immediately below the grafts) in doses totalling about 2ml/mouse. The possibility that the serum was « inactivated » in these experiments by the homologous cells known to be dispersed throughout the organs of hosts made tolerant by intravenous neonatal injection (Billingham and Brent[1, 11]) is at present being looked into, so is the question of whether sera from adjuvant sensitised mice have detectable titres of haemagglutinating antibodies.

In conclusion, using the accelerated destruction of skin homografts as the chief criterion of successful passive transfer of immunity, we have been unable to confirm the results obtained by Stetson and Demopoulos in the mouse. The reasons for this discrepancy are at present entirely obscure.

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We should like to thank Dr C A Stetson for his kindness in making available to us details of his experimental technique

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DISCUSSION

GORER I think first of all I would like to congratulate all concerned over this fine piece of work There are one or two other remarks I might make One is on the use of adjuvants. We have never used adjuvants by the actual technique of Stetson but we have used Freund's adjuvant with subcutaneous inoculation of various sorts of cells and get a worse antibody response than if we don't use adjuvants. We have used not only Freund's adjuvant but Pertussis vaccine as well with much the same result Now Dr Brent raised the question why do these types of homograft differ so much from one another ? Well there are probably several reasons. One is of course the susceptibility of the cells to antibody if you take a leukemia like EL4 which is highly susceptible the only result you can get by passive administration of serum is passive immunity if you take a tumour like sarcoma 1 which Mitchison worked with you could only get enhancement Now sarcoma BP8 is of intermediate susceptibility and different doses of serum give different results—a technically important point which arose in some work I did with Dr Kaliss. I will leave out the effects of intermediate doses and concentrate on the high and low doses. With a high dose of serum and an intramuscular graft of the tumour you do not destroy the tumour what you do get is mitotic arrest and the tumour hardly increases in size and sometimes never grows at all but the animal becomes actively immunised by the tiny graft which is left and so you never get a palpable lump. But if you rechallenge these animals are immune Now the other effect you can get if you use sublethal doses of antibody is a very violent stimulation of mitosis with an enormously rapid growth rate Now that is an effect you would not see with any

normal tissue grafts but only with tumours. It does serve as a complication. Well the point is this that in trying for enhancement or some of these other effects, I think it is a good idea to watch your serum dosage you can overdo it perhaps, if you give too large a dose of serum and if your target is partially susceptible to antibody as this one is That is just the point I wanted to make.

STETSON Of course, I am sorry to hear that Dr Brent has not been able to confirm our findings with adjuvant induced antisera Perhaps the discrepancy between his results and ours will turn out to be due to differences in grafting technique, survival scoring adjuvant preparation or other experimental variables We have recently had an opportunity to test the effect of our antisera on the skin grafts of tolerant animals (in the same strain combination CBA to A) and have had the same experience that Dr Brent has had, namely that the sera have produced no demonstrable effect on the grafts. We have found consistently however that it has been difficult or impossible to affect established homografts in non-tolerant animals with these immune sera, while we have had reasonably good luck in preventing the initial take of such grafts. The border line effects produced with sera against isologous cells which Dr Brent reports have not turned up in our experiments thus far but we shall certainly look for them.

BRENT I do think it is very important to stress that the criteria for the successful transfer of immunity used by Dr Stetson and by us, have been rather different We have used the degree of epithelial survival of the homograft six days after transplantation and this we have always found to be a very good baseline for experiments on immunity Dr Stetson has used rather different criteria, and I think it is important to bear that in mind It may be that grafts which are undergoing vascularisation are particularly susceptible to some kind of non specific interference or to interference by an auto-antibody this is suggested, rather feebly by our isologous control experiments.

VOISIN Perhaps Dr Woodruff would like to comment about the « adaptive period » during but not after which the serum could be active

WOODRUFF I have made a note to add a comment on this when I present my paper in a few minutes time. There is perhaps one other comment, though and that is on this question of adjuvants. My colleague, Dr Howard is working on what he calls a « built in » adjuvant which he achieves by infecting the animal with BCG We are interested to see whether we can repeat Barrett's work and produce transplantation

immunity with red cell antigens. Our original notion was to see whether Freund's adjuvant would help but it seems to me that this idea of Howard's of a built in adjuvant might be helpful and might be useful in other contexts also.

OWEN I might mention that when Dr Brent was in my laboratory two years ago we made rather strenuous efforts to produce second-set responses in mice by the injection of pure red cells after careful removal of the whites, using Freund's adjuvant and we were completely unsuccessful in these attempts. As I understand it Dr Brent your delayed skin tests have always been done within a species, within the guinea pig or within the rabbit Have you tried taking sensitised cells from a mouse (sensitised to mouse tissue) together with mouse antigen to see whether the guinea pig can provide a vehicle for revealing a reaction to materials taken from another species The guinea pig will become sensitive to anaphylactic sensitivity when provided with antibodies from many other species

BRENT No we have not done the kind of experiment which you have suggested It is indeed a very interesting possibility

LOUTIT Changing the subject a bit the thing that fascinated me was the link with Hašek's experiment this morning provided by the tolerant A line mice which were not chimeras. I wonder if Brent could elaborate on the test for negativity of chimerism in these cases

BRENT The test which Billingham and I have used on a great number of tolerant animals and in many different strain combinations is based on Mitchison's biological test for chimerism. This simply consists of killing the tolerant mouse removing the organs which are to be tested making cell suspensions from them and injecting these into animals isologous with the tolerant host. A few days later a donor strain skin graft is transplanted to the injected animals. If donor cells are present in the organ under test, then one would expect them to sensitise the normal host recipients against donor strain antigens and the skin test grafts should therefore break down very much more rapidly than grafts on normal non-sensitised individuals. I think I am right in saying that in every animal tested by us we have found evidence of chimerism in a whole variety of organs, from spleen and liver to blood thymus bone marrow and kidney Now the mice referred to by Dr Loutit were tolerant A line mice which as I have just said should have contained CBA cells throughout their tissues but which after injection with large doses of « Stetson serum » gave no evidence of chimerism (Their CBA

skin grafts were unaffected. If this *can be confirmed* our observation would fit very nicely into Dr Gorer's concept that cells of the lymphoid series are especially susceptible to serum antibodies and it might turn out to be a nice demonstration in one and the same animal that lymphoid cells are susceptible but that solid skin grafts are not. But we shall have to wait and see.

LOUTIT: Could I ask: does not this test merely indicate that the foreign element of the chimera is just below a critical level? It does not prove their absence, it just indicates that their numbers are below a threshold.

BRENT: Yes, that is very true. I think it would be unjustified to conclude that there is no chimerism at all. But, on the other hand, the number of cells required to sensitise an adult mouse is very small indeed: a dose of say 250,000 cells would be expected to register a very definite effect. If an animal is chimeric at all that is probably not an unreasonable number to expect among the cells in say a third of the spleen.

MEDAWAR: I should like to ask Dr Stetson if his technique of grafting was such that his results could be explained by a true serum transfer of a cytotoxic action against vascular endothelium, so preventing the primary vascularisation of his grafts. If that were so it would indeed be a somewhat different phenomenon from the one which we study by our particular technique.

STETSON: Yes, the effect which we are studying seems to be just that. In the « white graft » reaction of actively or passively immunized animals, there is apparently none of the initial vascularisation of the graft as seen in the usual « first set » and « second set » phenomena. Sera producing this effect may well do so by damaging endothelial rather than epithelial cells.

I might say that we have had a rather different notion to explain the failure of our sera to affect tolerant animals' grafts. Dr Brent has pointed out that these animals are chimeric, and it is just possible that the majority of cells required to effect solid homograft rejection are of the donor type. An antiserum directed against the graft would also therefore be directed against these effective cells which may be, as Gorer suggests, particularly susceptible to cytotoxic antibody. If the homograft reaction requires graft, antibody and effective cell then our serum treatment of tolerant animals may be supplying them with one essential reactant (antibody) but depriving them of another (effective cells).

VOISIN Dr Brent's paper is a very nice piece of work indeed and emphasizes that it is possible for a homograft to induce a delayed type of hypersensitivity On the other hand we know perfectly well that antibodies are under certain conditions, able to destroy a graft. For instance we showed that it is possible to kill a homograft rapidly or even to kill an autograft by injecting intraperitoneally a specific hetero-antiserum This is, of course, a highly artificial situation. The situation becomes less artificial when using iso-antisera, as we did in some cases obtaining them by grafting several primary recipients and taking their sera after a fortnight or as Stetson did by individual immunization by means of spleen cells plus adjuvants. Dr Gorer's results with cytotoxic antibodies are very significant also And I need not remind you of Billingham's work in which he showed that a short incubation of epidermal cells with a specific homologous antiserum (obtained by a simple skin graft) prevented them from growing when put back on the original donor Furthermore enhancement is an immunological phenomenon mediated by serum and this might play a part much more important than is usually thought in normal tissue grafts.

What I am leading up to is by no means to say that homograft rejection is a serological phenomenon but merely this a homograft is a very complex living material not comparable to any simple antigen not even to a bacterium it is able to elicit several types of reactions in its host. Some of these reactions have nothing specific about them in the immunological sense some are specific, and among these some consist in building up antibodies which can act in different ways, some consist in building a state of delayed hypersensitivity Now it is hard to believe that the behaviour of a homograft should not be affected by all these factors combined but by only one Its behaviour seems to me to depend on an interplay of all these factors, some of them being able to predominate under some conditions others under other conditions.

KROHN You suggested that cortisone was rather ineffective in your reaction in the guinea pig and that this might indicate a difference between your reaction and the tuberculin type delayed reaction I think however that the guinea pig is a very poor responder to cortisone and that people who have tried the effect of cortisone on the tuberculin reaction in guinea pigs have also been rather unsuccessful and prefer to use rabbits. So there may not be a difference after all

BRENT Yes, that may well be so. But I believe that the tuberculin reaction in the guinea pig can be at least partly suppressed I am aware that the rabbit is a much better animal from this particular point of view and we should certainly see if in the rabbit cortisone will suppress the transfer reaction completely

Evidence of adaptation in homografts of normal tissue

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Some years ago the writer (Woodruff [13, 14]) enunciated what he called the hypothesis of the critical period in the life history of homografts, according to which some types of homografts when once established become progressively less vulnerable to immunological attack and after a certain critical period are capable of surviving in the face of a degree of resistance in the recipient which they would not have been able to withstand at an earlier stage. This hypothesis implies that the grafts undergo some kind of adaptation, and the main object of this paper is to review the evidence for and against the claim that adaptation can in fact occur. It is on the evidence that the hypothesis must be based and not on our ability to suggest how the adaptation is brought about, but if the evidence appears convincing or even suggestive, then the question of possible underlying mechanisms is clearly a proper one for investigation.

Evidence of adaptation

As a rule homografts of normal tissues are destroyed too rapidly for any evidence of adaptation to appear. At the other extreme with a homograft which does not evoke any resistance—for example because the donor possesses no histocompatibility genes absent from the host or the host has been made completely tolerant of donor tissue—adaptation either will not occur or is likely to be difficult or impossible to detect. It would seem desirable therefore to look in the first instance for situations in which homografts survive for a long time or even indefinitely despite the fact that they do evoke an immunological reaction in the host. Such situations may arise in various ways, for example (a) when the only transplantation antigens which are present in the donor and

absent from the host are few in number and but weakly antigenic (b) when the graft is located in a specially favourable site such as the anterior chamber of the eye (c) when the dose of foreign tissue is extremely small (d) when the host is partially but not completely tolerant of donor tissue.

It is pertinent to ask also whether adaptation—though of a rather different kind—occurs when immunologically competent cells are transplanted homologously and are accepted by but initially at any rate react against the host.

Near-compatibility of donor and host

In 1947 Longmire, Stone Daniel and Goon reported a remarkable case of a human patient who received simultaneously skin homografts from many different donors. After 6 weeks only one graft remained alive and a second graft from the donor of this surviving graft was transplanted to a previously ungrafted area. The second graft took but began to ulcerate after 9 days. After 15 days it was completely destroyed and only then did the first graft show any signs of reaction

It should be possible with the inbred strains now available to reproduce this kind of situation in mice and study it extensively under controlled conditions. In particular an attempt might be made to produce a donor host combination differing only at one of the « weak loci » such that a first skin graft would survive apparently unchanged for months, and see what happened when a second graft was made during this period.

Homografts in special sites

It has long been known that homografts in the anterior chamber of the eye sometimes survive for many months or even indefinitely Sometimes this appears to be due to the fact that the graft does not become vascularized (Medawar [11]) and hence enjoys the same kind of isolation from host cells as a graft in a diffusion chamber (Algire Weaver and Prehn 1954 Woodruff [17]), or the mammalian foetus in utero (Woodruff [19]) Sometimes however a homograft in the anterior chamber continues to survive long after it has become vascularized from vessels in the iris.

Woodruff and Woodruff [21] investigated this phenomenon using homografts of thyroid in guinea pigs drawn from a mixed population They found inter alia that

(1) Thyroid homografts in completely thyroidectomized recipients commonly became vascularized and survived indefinitely in the anterior chamber (38 of 51 = 75 per cent appeared histologically normal after periods ranging from 30 to 424 days) whereas they were usually rapidly destroyed when placed in a subfascial or subcutaneous site (2 of 37 = 5 per cent appeared histologically normal after 30 days)

(2) A subcutaneous thyroid graft, made at the same time as, or a month preceding, a thyroid graft to the anterior chamber of the same (thyroidectomized) recipient from the same donor significantly reduced the chance of survival of the anterior chamber graft.

(3) A homograft to one anterior chamber of an intact (non thyroidectomized) recipient—which was slowly absorbed in accordance with Halsted's principle—rendered the opposite anterior chamber completely resistant to a further graft from the same donor even though thyroidectomy was performed in the meantime.

(4) A graft in the anterior chamber of a thyroidectomized recipient which had become vascularized and had survived for 6 months showed no change when the host received a subcutaneous graft from the same donor whereas the subcutaneous graft was completely destroyed

(5) In a significantly high proportion of cases a homograft which had been established in the anterior chamber for 3 to 6 months could be successfully transferred to a subcutaneous site and would there become vascularized and survive indefinitely

It was concluded that, under the conditions of the experiment the state of immunity evoked by a subcutaneous homograft extends to the anterior chamber and that a homograft in the anterior chamber is initially vulnerable to immunological attacks but ceases to be so at a certain stage in its life history

Another site in which homografts are specially privileged is withi the cornea and this as Billingham and Boswell showed holds good n only for orthotopic grafts of cornea itself but also for heterotopic intr corneal grafts Here absence of vascularization does appear to be necessary condition for homograft survival though somewhat surpri ingly it is not always a sufficient condition at any rate as far as orth topic corneal grafts are concerned Maumenee (Mueller and Maum nee [13] Maumenee [9]) in studying this phenomenon observed that corne homografts in rabbits which normally remained clear in 50 per cent c recipients became cloudy if skin was grafted from the same donor t the same recipient two weeks after the corneal grafting operation. O the other hand transplantation of skin from the same donor six week or more after corneal grafting did not cause clouding suggesting tha some form of adaptation occurs in corneal grafts during this interval

Low dosage homografts

It has been shown by Medawar[1] in rabbits that within limits the period of survival of a skin homograft varies inversely with the amount of foreign skin transplanted and the question arises as to what happens if the dosage is made extremely small. Does the survival time increase indefinitely or tend to some definite limit? There does not appear to have been any systematic attempt to find the answer to this question but Billingham and Medawar[2] experimented with very low dosage grafts in their studies on pigment spread in guinea pigs and found that whereas pigmentation could not be induced by orthodox homografts of pigmented skin it occurred regularly if homologous pigmentary dendritic cells were seeded in sufficiently small dosage on to a shallow recipient area in which the foreign cells had direct access to the bases of the hair follicles. Moreover pigmentation once started not only persisted indefinitely in a high proportion of animals but increased in area by the phenomenon of pigment spread. It is open to question whether this represents permanent survival and reproduction of homologous cells or as Billingham and Medawar argued an infective transformation of host cells. It seems clear however that the pigment forming complex—whether cellular or sub-cellular—possessed donor type antigenic specificity since the pigmentation could be bleached out by giving the recipient a massive skin graft from the original donor. Sometimes, however the bleaching was only temporary despite the fact that the massive skin graft was destroyed and it is an open question whether bleaching could be produced at all if the interval between seeding of pigmentary cells and orthodox grafting were sufficiently prolonged. Billingham and Medawar actually produced bleaching after an interval of 51 days and assumed that it could be produced « at any stage » but now that it is known that under certain conditions a homograft may survive for many months only to be rejected in the end our concept of what constitutes a long time in homograft evolution has changed. It would seem worthwhile therefore to repeat Billingham and Medawar's experiment but with intervals of up to a year or more between seeding of the pigmentary cells and transplantation of the massive graft, because if adaptation does occur at a cellular level such a system would seem likely to reveal it.

Partial tolerance

Cannon has drawn attention to the fact that in the experience of himself and his colleagues homografts of skin interchanged between newborn chicks—which survive permanently in 5 to 10 per cent of hosts

e sometimes rejected as late as 2 months after grafting but never 3 months. Cannon attributes this apparent invulnerability of grafts h are 3 months or more old to adaptation and, as evidence that the ige occurs in the graft rather than the host, points out (a) that not e than 50 per cent of homografts made soon after hatching can n grown to adult age, be returned to the donor without evoking a tion and (b) that adult hosts bearing surviving homografts which received soon after hatching almost invariably reject a second graft 1 the same donor but that the original grafts remain apparently hanged during and after the time when the second grafts are being cted.

Cannon s conclusion has been criticised on the ground that adult ken skin differs from new-born chicken skin in various ways and ht in consequence behave differently when grafted. This objection not however explain the writer s observation (Woodruff and Simp- [18] Woodruff [17]) that a homograft of adult rat skin which has been blished for several months in a host made nearly but not quite ipletely tolerant by injection in early life of cells from the prospec donor may apparently persist indefinitely while a second graft from same donor is rejected, sometimes in less than 2 weeks.

Against these findings may be set the observations of Medawar and colleagues that specific immunological tolerance in mice can be lished, and an apparently well-established homograft of skin (Billing 1 Brent and Medawar [3]) or adrenal (Medawar and Russell [12]) made breakdown by transplantation of lymphoid tissue from an animal enic with the host especially if this animal has been immunized inst the cells of the graft donor Once again however everything y turn on the length of time the initial graft was left in place before rance was abolished and in the writer s view this interval should be ended to at least 6 months and preferably 12 months (*) before draw any conclusions as to whether or not adaptation of the kind under ussion can occur in the grafts.

What may prove to be another instance of not-quite-complete tole- ce resulting in persistence of a first graft associated with rejection of cond one from the same donor is at present under investigation by writer The subjects of the investigation are a pair of human non ntical twins who were reported to be blood chimeras by Booth Plaut, nes Ikin, Moores Sanger and Race [4] When the condition was recog ed the twins were 21 years old. 86 per cent of the male twin s ery ocytes were group A and 14 per cent were group O whereas 99 per t of the female twin s erythrocytes were group O and 1 per cent were

(*) These figures refer to experiments in rodents.

group A. Moreover polymorphs showing female sex chromatin were present in the blood of the male twin in a proportion compatible with a mixture of male and female blood in the proportion shown by the blood groups.

Just over a year ago the twins generously allowed the writer to interchange small (1.0 × 1.0 cm.) full thickness skin grafts between them. These remained supple and unchanged in size for 12 months after which about one third of each graft with some host skin was excised for histological examination At no time was there any scabbing or ulceration but for the first six months the graft on the female twin appeared more red than the surrounding skin. Histological examination of the biopsy specimens has not revealed definite evidence of a homograft reaction but sex chromatin studies suggest that nearly all of the cells in the graft on the male twin have been replaced by cells from the host. The state of chimerism however had persisted, and differential agglutination tests (Mollison and Race, personal communication) show ıt 17 per cent of the red cells of the male twin are group O and about 2 per cent of the red cells of the female twin are group A (*). It is difficult to compare skin grafts with grafts of haemopoietic tissue but the findings suggest that while the blood chimerism is stable the male twin is not completely tolerant of his sister's skin.

Blood chimerism though rare in man is common in cattle It has been reported (Anderson Billingham Lampkin and Medawar [1]) that cattle twin chimeras are completely tolerant of each other's skin but in view of the above findings it has been thought worth while to re-examine the question. The writer has been fortunate enough to locate a group of quadruplet cattle in which Dr Lampkin interchanged skin grafts several years ago and it is proposed to examine the grafts macroscopically and microscopically check the blood grouping and if possible make a second-set reciprocal skin grafts. Unfortunately however as far as is known it is not possible to use Barr's technique of chromatin sexing in cattle.

Transplantation of immunologically competent cells

The occurrence of *runt disease* and *secondary disease* following transplantation of immunologically competent cells to immunologically immature animals and heavily irradiated animals respectively is widely if not universally attributed to an immunological reaction of the grafted

(*) Dr Mollison states that the figure of 17 per cent is based on counting the figure of 2 per cent is based simply on comparison with known mixtures. The latter estimate was however confirmed by labelling the whole sample with ^{51}Cr and then lysing the A cells with potent anti-A serum and measuring the amount of ^{51}Cr liberated This gave a figure of 2.6 per cent group A cells in the female twin.

cells against the host. It seems clear moreover that animals *may* recover from these conditions and remain chimeras (*) The question arises whether these recovered animals are ever not just chimeras but *chimeras in respect of immunologically competent cells,* because if so it would seem to imply that the cells in question have undergone some kind of adaptive change. It would not be a matter simply of cells presented with a gigantic quantity of antigen failing to react in a normal way but rather of cells reacting for a period of weeks and then gradually ceasing to do so The whole question of the immunological status of chimeras merits much more study than it has yet received and the question propounded above might form one useful starting point.

The nature of the adaptation

It is conceivable that in some instances, what appears to be evidence of adaptation is in fact the result of total replacement of the cells of a homograft by host cells, but in many of the examples cited this is clearly not the case.

In genuine cases of adaptation of homografts of normal tissue (**) if they exist, there would seem to be two possible explanations (a) replacement of certain elements of the graft for example connective tissue stroma and vascular endothelium and (b) some change at a cellular level.

Thyroid grafts in the anterior chamber of the eye characteristically undergo central necrosis followed by regeneration from surviving cells at the periphery and the same may well be true of grafts of other endocrine tissues in this situation. It seems likely that, as a result, the stroma of a graft of long standing is of host origin A hint that this may be significant in the present context is perhaps provided by the observation of Medawar [11] that homografts of skin in the anterior chamber survive only if they remain avascular for they do not undergo a cycle of necrosis and regeneration Against this it may be urged that homologous epidermal cells seeded on to a surface defect which by definition do not possess a stroma of their own are soon destroyed (Billingham and Sparrow [4]) but the comparison is hardly fair because the epidermal cells would seem to be much more exposed to contact with immunologically active host cells than are the epithelial cells of a regenerated thyroid graft

(*) This is true even of radiation chimeras in which the foreign cells are heterologous (see Makinodan and Anderson Welling, Vos and van Bekkum)

(**) The possibility of selection, which would have to be considered in a discussion which included homografts of neoplastic tissue would not seem likely to arise with normal tissue

Stromal replacement may account also for some other instances of adaptation but it would seem unwise to continue to ignore the possibility of adaptation at a cellular level. This need not necessarily imply a change in nuclear antigenic structure the antigens might for example simply become less accessible though until we begin to know something about what might be termed the final common path of the homograft reaction—the life and death struggle between activated host cell and foreign cell—it would be unprofitable to speculate about how such a change could be brought about or what it would entail in terms of cell structure. But even the suggestion of a change in antigenic—and genic—structure cannot in these days be ruled out *a priori*. Indeed if genetic changes comparable to those which can be produced in bacteria by exposing them to purified DNA or to bacteriophage can occur in mammalian cells, it seems likely that they will arise in biological systems in which cells of different genetic constitution are brought into close contact rather than be produced by injecting DNA into animals with a syringe.

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DISCUSSION

MERRILL. I would like to make one or two po ... question if I may I think it is certainly well when ... to keep in mind the chimera but I would like to ...

that the chimera—at least the one that was slain by Bellerophon—was part lion part dragon Now if we say that Jack was 86 % lion and 15 % dragon he was certainly a true chimera If on the other hand he had lion s ears, tail paws and teeth and so on down through all 23 known characteristics of the lion he is not a chimera but a lion. This is probably important in consideration of the twins we discussed this morning I am also extremely interested in the problem that has been raised and perhaps will be elaborated by Dr Medawar about the dose phenomenon in terms of homograft response. Dr Medawar stated in his protocol that the dose-response was discouragingly flat, and yet insofar as the persistence of a homograft goes as Dr Woodruff suggested it may be that very small doses may result in persistence. I would like very much to know the answer to that.

SIMONSEN Dr Woodruff mentioned the cases of irradiation chimeras which passed through a state of secondary disease, but recovered. I think that in some cases the basis of the phenomenon (I shall present evidence of it tomorrow) is that host cells recover and manage to reject the graft, and during that process the animal may become sick but recover again at a later stage. But another possibility which has not been proved to my knowledge, but perhaps could be proved, is that the donor cells are of two kinds which behave differently Those which were immunologically mature at the time of transplantation might succumb to an immunological reaction between their own antibodies and the antigens of the host while other members of the donor cell population which were immunologically sufficiently immature at the time of grafting might become tolerant instead of immune. By the immunological pressure exerted in this way the immature cells would stand a better chance of survival than the mature cells. The problem then is to check if cells of donor type survive which are tolerant or at any rate non reactive towards antigens of the host, but are otherwise fully reactive. And this, I think, can be done. The procedure, I shall mention in more detail tomorrow but I mention it in this context too

Suppose you irradiate a mouse A and graft bone marrow from a foreign strain B The A mouse passes through a phase of secondary disease and then recovers. The problem now is to test if the A mouse contains B cells which are tolerant against A but fully reactive against a third strain which we call T The way I suggest requires an F_1 hybrid between B and T This hybrid should be some 4 10 days old If you graft, say spleen from the chimera into this hybrid, all the A cells are eliminated by a host versus-graft reaction because 4 10 day old mice are old enough to reject the A cells, while the B cells are accepted for gene

Stromal replacement may account also for some other instances of adaptation but it would seem unwise to continue to ignore the possibility of adaptation at a cellular level. This need not necessarily imply a change in nuclear antigenic structure the antigens might for example *simply become less accessible*, though until we begin to know something about what might be termed the final common path of the homograft reaction—the life and death struggle between activated host cell and foreign cell—it would be unprofitable to speculate about how such a change could be brought about or what it would entail in terms of cell structure. But even the suggestion of a change in antigenic—and genic—structure cannot in these days be ruled out *a priori*. Indeed if genetic changes comparable to those which can be produced in bacteria by exposing them to purified DNA or to bacteriophage can occur in mammalian cells, it seems likely that they will arise in biological systems in which cells of different genetic constitution are brought into close contact rather than be produced by injecting DNA into animals with a syringe.

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DISCUSSION

MERRILL I would like to make one or two points and ask one question if I may I think it is certainly well when one considers twins to keep in mind the chimera but I would like to remind Dr Woodruff

that the chimera—at least the one that was slain by Bellerophon—was part lion part dragon Now if we say that Jack was 86 % lion and 15 % dragon, he was certainly a true chimera If on the other hand he had lion s ears tail, paws and teeth and so on down through all 23 known characteristics of the lion he is not a chimera but a lion. This is probably important in consideration of the twins we discussed this morning I am also extremely interested in the problem that has been raised and perhaps will be elaborated by Dr Medawar about the dose phenomenon in terms of homograft response Dr Medawar stated in his protocol that the dose-response was discouragingly flat, and yet insofar as the persistence of a homograft goes, as Dr Woodruff suggested it may be that very small doses may result in persistence. I would like very much to know the answer to that

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tical reasons They will therefore be stimulated by the T antigens to produce a spleen and liver enlargement as I have earlier described as the graft versus host assay So by this means you can actually check if immunologically competent cells of donor type survive, and by another appropriate graft versus host assay you can assert whether or not they are tolerant against the host

MEDAWAR I should like to comment on two phenomena which have been or could be, described as a consequence of adaptation. One is a change in the antigenic phenotype of the cell surface Some recent work of Dr Toolan's on the apparently very long survival of homografts of early rabbit embryo skin could be explained in terms of some such adaptation. It is not enough to say that the embryo skin was immunologically immature, because if it did in fact survive as a homograft the antigens in it should have matured in due course and should have given rise to transplantation immunity There may have been a cellular transformation analogous to that which Sonneborn described many years ago in *Paramecium*, one which I think the Kleins refer to in their paper The second example I would like to comment on is only superficially similar to this one the apparent adaptation which Cannon and his colleagues recorded when newborn chick skin was transplanted to other newborn chicks. No this, I think is a totally different phenomenon. Billingham and Silvers have shown that adult chicken skin contains lymphoid cells and should therefore be able to produce a host versus-graft reaction, and Billingham and Silvers have proved by Simonsen's technique that that is in fact the case for adult chicken skin grafted on to the chorio-allantois will cause splenic enlargement Now if one transplants newborn chick skin as a homograft one will get a tolerance of the graft against the antigens of the host, and this seems to me to be an entirely sufficient explanation of why newborn chick skin should behave better as a homograft than adult skin So « adaptation » is by no means a homogenous concept and I think one should confine the use of the word to the phenotypic transformation of cellular antigens. I would very much like to hear Dr Klein's views on this.

KLEIN I rather hesitate to bring neoplastic tissues into this discussion but I think one might recall in this connection the phenomenon described by Barrett and Deringer which appears to be a rather clear case of adaptation This, as you may remember is the change that occurs in spontaneous mammary cancers after passage through F_1 hybrid hosts produced by outcrossing the strain of origin with another unrelated strain The tumours grow progressively in a certain percentage of F_2 hybrids. This percentage is rather characteristic for every tumour

and is usually interpreted in terms of Mendelian segregation ratios. One usually speaks about 3-gene requiring or 2-gene requiring tumours. This is not a very good way to express it but we may adopt this use for the convenience of reference. Barrett and Deringer have discovered that tumours may regularly diminish their gene requirements during F_1 passage. In this case, selection has been excluded and the change seems to be produced in the majority of the cells inoculated and at a rather early stage. It does not seem to involve any loss of the antigenic receptors of the cells because the changed and unchanged cells become entirely similar if they are compared in pre-immunised F_2 hosts. It seems as if the changed cells enjoy a privileged position towards certain component factors of the homograft reaction. The systems involved probably do not include H-2.

KROHN: Prof. Woodruff has suggested that autografts might be replaced under certain circumstances and that the process of replacement might be invisible. There is one situation that I know of where autografts are certainly not replaced, because they remain visible and that is when portions of the sexual skin of monkeys are transplanted from one part of the animal to another. If that is done the skin which has its own specific responses to oestrogen and its own particular coloration and so on remains entirely as it was in its original site: it never alters its epithelium does not alter and its stromal responses and its vascular responses, and its responses to oestrogen all behave entirely normally and as you would expect. This is true over a period of 6 or 7 years or so so that here at any rate the autograft has maintained its integrity.

VOISIN: I do not think Dr Woodruff feels very strongly about replacement of autografts.

HAŠEK: I should like to add a remark about Billingham and Silvers' explanation of Cannon's findings. Zotikov and Puza had the same experience—that adult chicken skin produced considerable enlargement of the spleen of the chick embryo. And they also find that the skin does not cause enlargement after irradiation with 1000 r. I think that to solve Cannon problem it would be useful to graft irradiated adult skin on newborn animals. As to the possibility of genetic adaptation of the mammalian cells, we studied in our laboratory some long term interspecific chimeras between chicken and turkey whose erythrocytes clearly contain both chicken and turkey antigens. In a one year old turkey chimera which had more chicken than turkey erythrocytes the blood cells were exposed to specific anti-chicken antibodies. The agglutinated

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erythrocytes were separated from the non agglutinated by differential centrifugation and the washed clumped erythrocytes were mechanically broken up we thus obtained a suspension of turkey chimera erythrocytes, containing chicken antigens. An attempt was thereupon made to demonstrate turkey agglutinogens in these cells by absorption of chicken immune serum against turkey blood. Agglutination did not work but the erythrocytes were sensitised by gammaglobulin. These erythrocytes did not however reduce the agglutinin titre. Erythrocytes containing both turkey and chicken antigens simultaneously were therefore not demonstrated in the blood of these chimeras. In plants, Winkler has demonstrated with a wealth of material two cases of real vegetative hybrids, perhaps the result of nuclear fusion. The occurrence of true somatic hybrids in mammalian cells is certainly low but where cells of different genetic types coexist in intimate relationship the possibility deserves more attention.

Thirdly I completely agree with Dr Woodruff that the situation of the tolerated homograft is very complicated. These complications are perhaps not always fundamental, as the following example will show A tolerant white turkey was grafted with chicken skin bearing black feathers. The heterograft was tolerated for three months. At first the feathers grew black and in graft-specific orientation but after a month only white feathers grew (we forgot to examine for the presence of pigment granules in the feathers). But it seems to us probable that the phenomenon was due to the fact that pigmentary cells are more sensitive to immunity than the epidermal cells and that that is why feathers without pigment grew on the recipient.

Transplantation immunity and immunological tolerance and the study of antigenicity of tissues and their derivatives

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The study of antigenic differences between donor and recipient which are responsible for tissue incompatibility in homotransplantation is one of the most important problems in transplantation research. Since tissue incompatibility began to be explained as an immunological phenomenon the question arose as to what antigen in the tissues produces the antagonist reaction in the recipient, in which tissues it is present and in which it is not how stable it is, what chemical substances are involved etc. Chemical characteristics distinguishing each individual of the same species were designated by Loeb as individuality differentials (Loeb [30]) Various kinds of differentials (species, order class differentials) including the individuality differentials comprise the organismal differentials. Blumenthal [7] demonstrated that α transplanted pieces of tissues and organs give off substances which bear the organismal differentials which not only diffuse into the areas of the host directly around the graft but in addition reach the general circulation thus they may be carried to distant places, presumably to the leucocyte production organs and there exert an effect on the number and kind of leucocytes which circulate in the blood [8] The mode of reaction especially of the lymphocytes served as the finest indication of individual differences as represented by the individuality differentials of host and graft. He assumed that individuality differentials were presumably of a protein nature because all the known agents causing changes in the constitution of proteins produced changes in organismal differentials [8] The following-up of the lymphocytic reaction in the circulating blood however has not been worked out by other authors the substances responsible for the individual differences have, therefore not been isolated and until recently our knowledge of them was greatly limited. Medawar and his associates taking advantage of

erythrocytes were separated from the non-agglutinated by differential centrifugation and the washed clumped erythrocytes were mechanically broken up we thus obtained a suspension of turkey chimera erythrocytes containing chicken antigens. An attempt was thereupon made to demonstrate turkey agglutinogens in these cells by absorption of chicken immune serum against turkey blood. Agglutination did not work but the erythrocytes were sensitised by gammaglobulin These erythrocytes did not however reduce the agglutinin titre. Erythrocytes containing both turkey and chicken antigens simultaneously were therefore not demonstrated in the blood of these chimeras. In plants Winkler has demonstrated with a wealth of material two cases of real vegetative hybrids, perhaps the result of nuclear fusion. The occurrence of true somatic hybrids in mammalian cells is certainly low but where cells of different genetic types coexist in intimate relationship the possibility deserves more attention.

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lan[11] showed the permanent survival of rabbit foetal homografts which promised to be applicable in man Together with J Chutná we investigated whether mouse embryonic tissues were capable of surviving in mice of another inbred strain or whether they behaved like adult tissues.

Embryos 8 10 12 days old of C57 Black mice were isolated cut into several fragments and transplanted under sterile conditions to a vascular bed on the back of adult mice of the inbred A strain. They were then sprinkled with crystalline penicillin, covered with sterile silk impregnated with lanoline and protected with a plaster bandage. Survival of the embryonic grafts was checked macroscopically and histologically for 20 days after transplantation. The antigenicity was evaluated using the transplantation test (Billingham et al.[2]) and the cytological test (Chutná[10]) for transplantation immunity Four to five days after injection of embryonic cells skin from the back of adult mice of strain C57 was grafted on to the recipients and isolated epidermal cells from C57 mice, obtained by digestion of the epidermis in 3 % crude trypsin solution (Billingham and Sparrow[4] Weaver et al[26]) injected subcutaneously At the end of the sixth day after transplantation grafts were evaluated histologically as compared with a positive and a negative control The positive controls were grafts which were treated in the same way and were applied to recipients in which one skin graft had already regressed while the negative controls were grafts applied six days previously to non immunized recipients. Skin was also collected at the end of the sixth day from the back at the site of implantation of the epidermal cells, and after two hours fixation the areolar connective tissue was treated in the form of Jasswoin preparations. Like the histological sections of the grafts, these were stained with haematoxylin eosin Survival of the epidermal cells and the intensity of the local cytological reaction were evaluated quantitatively by the differential cytological pictures. The results of the evaluation were again compared with a positive and negative control.

A macroscopic and histological study of embryonic grafts from 8 10 12 day old mouse embryos showed that the maximum time of survival was 16-18 days (tab I) In most cases only cartilage was still undamaged 25 days after transplantation The histological study showed that destruction of embryonic grafts was accompanied by the same local reaction as in the destruction of adult skin grafts.

In the transplantation immunity test complete destruction of the epithelium of the test graft was found in recipients which four days before transplantation had received an injection of embryonic cells of different ages (tabl II) or an embryonic graft while in non immunized animals the epithelium survived completely Subcutaneously injected

r experiences with skin homografts between inbred strains of mice[4] : able to develop a delicate test for transplantation immunity which le it possible to study the nature of these so-called « transplantation gens » and their occurrence.

By means of this test they observed the antigenic properties of the ear tissue fragments, affected by various mainly enzymatic treat ts (Billingham Brent Medawar[2]) and especially in their most recent , they studied the antigenic activity of subcellular material from mouse spleen Their results suggest that the antigens are probably lar to the blood-group mucolds (Billingham Brent Medawar[3]). The ity of their preparations can be *destroyed completely by the extracts* ined from Trichomonas foetus.

Chutná[10] in our laboratory worked out a cytological test for trans tation immunity which consists in evaluating the degree of lym :ytic infiltration around the inocula of epidermal cells for determin 1 of transplantation immunity In our work to be described, we used tests in parallel with concurring results The second test recently *by Billingham Brent and Medawar*[3] on *guinea-pigs previously sen* :d by skin homografts also appears to have a chance of success in re studies. Though the transplantation antigens at first seemed : substantially different from antigens causing the enhancing effect ppears now that they have much in common or even are identical iat it may be possible some time in the future to make use of the m employed in the study of antigens inducing enhancing effect *.andutsch and his co-workers*[11] *for a special study of transplants* antigens. We also plan to examine the relationship between these gens in our laboratory

One way to the study of antigenic power of tissues with conclusive ts is that of inducing immunological tolerance From the survival of the test graft in animals injected in the adaptive period with e material from the same donor it was concluded that the same iplantation antigens were also present in *other tissues of the same* idual i.e. in the spleen kidney leucocytes but they were not found rythrocytes (Billingham Brent Medawar[1] Hasek Lengerová :rnová[14])

In my paper I wish to present some recent results that we have ned in experiments using the previously mentioned tests

Transplantation immunity tests and homotransplantation of embryonic tissues

Recent studies have been concerned with problem of the extent to h *embryonic tissues* antigenically mature The results of H Too-

Cases of permanent survival of embryonic grafts reported in the literature are evidently associated with the exceptional transplantability of certain tissues, e.g glands or with particular body sites, which for some reason or other are not suitable for inducing transplantation immunity The positive results of Snyderman with skin grafts could probably be explained on the basis of non-specific depression of the reactivity of recipients afflicted with neoplastic disease, since Rogers[23] reported negative results in normal patients, like Goldstein and Baxter [12]

Test for transplantation immunity and antigenicity of cell free material.

The transplantation immunity test could be no doubt a very delicate method convenient not only for the study of the antigenic activity of cells, but also of cell-free material Billingham Brent and Medawar [8] described the extraction of antigens causing transplantation immunity In order to induce transplantation immunity in mice we use a simple crude aqueous extract which is obtained by mashing spleen with sand in cooled distilled water under sterile conditions at pH 6.8 7 0 followed by shaking the homogenate for 3 12 hours and by centrifugation at 18,000 g for 30-60 minutes. Our cell free extracts show varying ratio N/P maximum UV absorption at 258-260 mμ, approximately 12 5 mg of proteins in 1 ml 0 125 mg of lipids in 1 ml a complete spectrum of free amino-acids and chromatographically detectable traces of monosaccharides in the hydrolysate which have not hitherto been identified This extract was injected intraperitoneally into mice of another strain and grafted by the method of Billingham Brent and Medawar [3] Six days later the grafts were removed for histological examination. Survival of the epithelium of the test graft was again evaluated i e. compared with positive and negative controls as in experiments with embryonic tissues.

The results show that crude aqueous cell free spleen extracts from one strain of mice are capable of producing transplantation immunity in almost 100 % of the animals of a second strain which can be tested by destruction of the test skin graft epithelium from mice of the donor strain. Most of the liver extracts of corresponding composition and concentration did not produce transplantation immunity (tab III).

Billingham Brent and Medawar [2] found in 1956 that the antigenic power of nuclear cell fragments to elicit transplantation immunity was destroyed by the action of deoxyribonuclease We thought it worth while to study the part played by deoxyribonucleic acid in transplantation immunity [14] The transplantation immunity test devised by the above-mentioned authors was again used together with their method of evaluation. DNA was prepared from the liver spleen and thymus of mice using the method of Zamenhof [27] The harmlessness of this

epidermal cells from the donors were destroyed destruction being accompanied by a lymphocytic reaction which occurred at the same time and was of the same intensity as in the positive controls In negative controls homologous epidermal cells survived in the subcutaneous tissues for 12 13 days.

TABLE I

Age of embryos in days	No. of recipients	Transplantation method.	Survival in days.	Test for transpl. immunity
8	6	I.M. implantation	12 — 14	
8	3	Transplantation of fragm. embryos to back	12	
8	2	»	12 — 16	+
8	5	»	12 — 14	+
8	4	»	12 — 13	+
12	5	»	12 — 18	

TABLE II

Age of C 57 embryos	No. of strain A recipients	No. of infected cells	Immunity test	
			transplant	cytological.
12 days	5	40 10^6	+	+
10 days	3	156 10^6	+	+
8 days	4	1,4 10^6	+	+
8 days	3	306 10^6	+	+

The results obtained in our experiments show that tissues of 8-day old mouse embryos i.e. the youngest embryos which it has hitherto proved possible to isolate satisfactorily did not actually survive longer than adult tissues and that they produced the same transplantation immunity as the grafts of adult tissues.

According to Toolan[23] 45 % of homografts from 12 13-day-old rabbit embryos survived in unconditioned adult recipients. Snyderman[21] repeated Toolan's work in humans. He transferred the skin obtained from embryos between two and four months of age to adult recipients most of them suffering from neoplastic disease. Two of the nine patients grafted have maintained the embryo grafts for nearly one year On the contrary Goldstein and Baxter[12] observed an active homograft reaction (rejection response) in 17 human recipients to whom skin grafts from foetuses between 17 and 33 weeks of age had been applied. The average survival period was 12.6 days.

The present results with inbred mice do not confirm the results of Toolan and Snyderman in mice Similar results with older mouse embryos were also mentioned by Billingham et al[3]

method was tested by demonstrating that it did not inactivate the pneumococcal transformation factor added at the begining of the preparation. It was therefore believed that this method should also preserve other biological characteristics of the deoxyribonucleic acid molecule The results showed that isolated deoxyribonucleic acid does not produce transplantation immunity comparable with immunity caused by a previous injection of donor tissue. Only in a few cases was it possible to observe the beginning of the destruction of the test graft. The causes of the weak reaction have not yet been revealed.

On the other hand the injection of DNK also did not produce prolonged survival of homografts between inbred strains of mice.

In this connection we also studied the reaction of recipients to heterologous deoxyribonucleic acid by means of serological tests. The deoxyribonucleic acid isolated from fresh calf thymus containing minute or small amounts of protein did not under our experimental conditions evoke a specific antibody response in rabbits detectable by the usual serological tests (complement fixation test precipitation, etc.)[17]

Test for induction of immunological tolerance and the problem of inducing tolerance by means of cell-free material.

The term « immunological tolerance » covers a number of phenomena, the common denominator of which is that they are produced by conditioning the recipient during the adaptive period by embryonal parabiosis, intraembryonal or postembryonal injections, grafts etc They include

1) prolonged or permanent survival of tissue homo- and heterografts,

2) complete or partial suppression of the formation of iso- or heteroagglutinins

3) complete or partial suppression of the formation of antibodies against pure protein antigens

4) survival of tumour homo- and heterografts in the recipient following the injection of the donor s normal or neoplastic cells in the adaptive period.

The general view is that, basically immunological tolerance is a deviation from the normal immunological response mechanism produced by exposure of young animals (or birds) to an antigenic stimulus, i e. a stimulus which when administered to normal adult animals, produces sensitization or immunity It may therefore, be presumed that there is a correlation between the ability to induce tolerance in very young animals and immunity in older animals.

TABLE III

Material injected	*Donor and recipient strains*	*No of mice*	*Mode of administration.*	*Mean value of N/P in the extract.*	*Mean amount of N in the inoculum (in γ/1 mL)*	*Histologic examination of the test graft (+ reaction indicates complete destruction of epithelium — reaction indicates appearance of epithelial cells as in first grafts)*
Crude water spleen extract	A-CBA or CBA-A	12	i.p	10 76	6.935	+ + + + + + + + + + + +
Crude water liver extract	A-CBA or CBA-A	17	i.p.	13 8	5.309	+ + ± ± ± — — — — — — — — — — — —
Control experiments						
—	A-CBA or CBA-A	11	—	—	—	— — — — — — — — — — —
Spleen cells	A-CBA or CBA-A	7	i.p.	—	—	+ + + + + + +

TABLE IV

Material injected	*Experimental birds*	*No. of birds*	*Mode of administration*	*Mean value of N/P in the extract*	*Mean amount of N in the inoculum (in γ/ml)*	*Survival of the test graft from the same donor*
Crude water spleen extract	chicks	19	i.v. injection of embryos	5.14	344	did not survive for 20 days
—	control chicks	17	—	—	—	did not survive for 20 days
Crude water spleen extract	ducklings	9	i.v. injection of newborn ducklings	12.8	812 (maximum amount of 2,500)	regressed completely by 30 days
—	control ducklings	16	—	—	—	regressed completely by 20 days

It may therefore be assumed that exposure of the animal in the adaptive period to inactivated transplantation antigens can under certain conditions lead to immunological tolerance to skin grafts from the same donor

The problem of inducing tolerance by cell free material was studied previously by Hašek et al [13] In their experiments they attempted to settle the question of whether it is possible to induce tolerance by a mere exposure of the embryo to a foreign antigenic material to which the embryo becomes metabolically adapted They used destroyed blood cells, but failed in every case to induce immunological tolerance. Billingham Brent and Medawar [3] also report that they have not been able to secure transplantation tolerance by injecting any of their preparations into newborn mice or newborn chicks.

In our laboratory we were also engaged in studies on this problem. We have tried with J Majer to establish whether cell free material containing non inactivated transplantation antigens was capable of eliciting immunological tolerance of skin homografts. Since crude aqueous spleen extracts have conferred transplantation immunity we used these extracts in order to secure transplantation tolerance

These experiments were carried out in chickens and ducks. Since no inbred strains were available skin from the original donor was kept in a glycerin medium in contact with CO_2 (Billingham and Medawar [3]). Tested material from one bird was administered intravenously to chick embryos (usually on the 13th day of incubation) in amounts of 0.1-0.2 ml In ducks, in which the adaptive period extends into postembryogenesis (Hašková [15]) one or two doses of extract was injected on the first days after hatching in amounts of up to 1 ml Test grafts from the original donors were applied to chickens 14 days after hatching when homografts normally never survive permanently (Cannon Longmire [9]), and to ducks 21 days after hatching In transplantation from adult donors, skin from the wattle or leg — the structure of which is different — was used The regression time was determined macroscopically

When crude aqueous cell free spleen extracts from adult chicken or duck donors were administered intravenously to chick embryos or newly hatched ducklings for testing immunological tolerance they never produced permanent or prolonged survival of skin grafts from the original donor in 14-day-old chicks or 3 week-old ducklings (tab IV)

Even when the extract mixed with the incomplete Freund's adjuvant was administered to 14-day-old chick embryos prolonged survival of test homografts did not follow

The results show that the antigens responsible for transplantation immunity are present in crude aqueous spleen extracts but attempts to

TABLE IV

Materia. injected.	*Experimental birds*	*No of birds*	*Mode of administration.*	*Mean value of N/P in the extract*	*Mean amount of N in the inoculum (in γ/ml)*	*Survival of the test graft from the same donor*
Crude water spleen extract	chicks	19	i.v. injection of embryos	5 14	344	did not survive for 20 days
—	control chicks	17	—	—	—	did not survive for 20 days
Crude water spleen extract	ducklings	9	i.v. injection of newborn ducklings	12.8	812 (maximum amount of 2.500)	regressed completely by 20 days
—	control ducklings	16	—	—	—	regressed completely by 20 days

induce immunological tolerance to skin grafts by non-cellular material capable of producing transplantation immunity in mice all bore negative results, even when the extract was administered to ducks in two injections on consecutive days. When repeated doses of aqueous spleen extract from chickens were injected into ducklings survival of chicken heterografts in ducks was not prolonged though spleen cells produced a threefold survival of heterografts. In our previous experiments we found however that immunological tolerance to foreign erythrocytes could be more easily induced by repeated smaller doses of erythrocytes administered in the adaptive period, than by single injections (Hašková [11]) The injection of isolated spleen cell nuclei also bore negative results although Billingham Brent and Medawar [2] demonstrated that these were capable of producing transplantation immunity

The failure of earlier attempts by Hašek [12] to induce tolerance by destroyed cells may have been due to inactivation of the « transplantation antigens » by freezing *In the present experiments, an immunity test* showed the presence of « transplantation antigens » in cell free extracts. It should be borne in mind however that the induction of immunological *tolerance requires the presence of the whole complex of « transplantation* antigens » There is a possibility that extract prepared in the way described above does not contain the whole antigen complex and that *certain antigens are only sufficient for producing immunity*

A further cause of failure to induce tolerance by cell free material could be that the dose injected during the adaptive period was insufficient. In addition to the experiments in chickens, therefore experiments were carried out in ducks, to which a large amount of material can be administered intravenously after hatching (the maximum amount of N in the inoculum per mouse was 2500 γ) *their results were still negative* Another very important factor in the induction of immunological tolerance is time (Hašková [11]). Repeated injections are more satisfactory than single injections. *It is possible that non-cellular material is* removed from the recipient's body before it has time to take effect In the present experiments however even repeated injections did not give better results.

Although one cannot exclude the possibility that better chemical preparation of the antigens which participate in transplantation immunity will eventually given a suitable administration method make induction of a state of immunological tolerance by cell free material possible we are of the opinion that a distinction should be made between immunological tolerance to skin grafts *and tolerance to pure antigens* Immunological tolerance to grafts has hitherto been produced only by living cells capable of further proliferation in the recipient's body It can also

be presumed that the formation of a cell chimaera, permitting the relatively permanent persistence of antigens in the recipient is a condition for the maintenance of immunological tolerance to transplantation antigens.

In this laboratory further investigations are being carried out to study the possibility of producing tolerance by cell free material, to study the stability of « transplantation antigens » their relationship to the enhancing effect as well as their nature. The results, which might have been presented here, are not yet available.

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DISCUSSION

PREHN. I would like to make a comment about Toolan's work. It seems to me quite probable that in the case of the survival of embryonic skin grafts in rabbits, her materials may be genetically relatively uniform, at least she had little control over this particular factor. It seems to me that if one wished to reproduce this type of result in mice it would be worthwhile to use mice which were genetically not too incompatible. The C57 mice that were used in the present reported experiments, if I remember correctly, are about as different genetically from most of the rest of the inbred strains as it is possible to get. It seems to me therefore that it would be advantageous to do this type of work in animals that were relatively similar, the genetic differences being minor, and it is then conceivable that one would see results somewhat similar to Toolan's.

MURRAY. I would also like to comment on the question of the antigenicity of the embryonic tissue, especially in relationship to Dr. Toolan's work. Dr. Hašková has already mentioned that her own work agrees very well with that of Drs. Baxter and Goldstein in humans. However, the observations of Sneidermann in the human apparently support the observations of Toolan. Personally I have great difficulty in accepting whole-heartedly any observation regarding survival of early embryonic skin in either man or the experimental animal. The bits of tissue are so small and fragile that objective certainty is almost impossible to obtain. The use of patients as hosts in terminal conditions further complicates the problem of evaluation.

Two additional bits of information lend support to the fact that embryonic tissue is antigenic. First, none of the vascularized foetal endocrine transplants have demonstrated objective evidence of survival after thorough follow-up study of patients. The patients usually feel

better but there is no indication that the tissue is surviving Secondly Dr K. Porter has in press a manuscript describing the use of embryonic liver in the rabbit in order to circumvent the secondary response. The period of gestation in the rabbit is 31 days. After using the embryonic liver tissue at 20 days, and at 27 days, and then one day after birth, the three periods of time arbitrarily selected, a small but constant percentage of hosts subsequently rejected the proliferating hematopoietic tissue. In other words, there was enough antigenicity in the embryonic tissue at all stages to initiate a rejection on the part of the host Incidently none of his rabbits developed secondary disease following use of the 20 day sample

BERRIAN I should reserve my comments on the completeness of antigenic extracts till tomorrow morning but as the question has already come up twice today as regards the ability of antigenic extracts to induce tolerance, I would like to say that I think the answer to this may depend on the fact that the extracts do not contain all the antigenic factors presented by living cells and the data I shall present tomorrow show this quite clearly This work involves only four strains of mice, but I think the results are probably representative of antigenic extracts prepared by the methods of the Medawar group

VOISIN I am not sure that it is possible to induce tolerance by means of non-living material There are two other possible explanations of the failure of extracts—for example the necessity for cellular survival possibly for a long time, and the fact that the cells which are able to induce tolerance are immunologically competent cells that is why I should like to ask Professor Medawar for some amplification of what he said very shortly this morning concerning monocytes and epidermal cells.

MEDAWAR Terasaki has shown that pure monocytes in chickens can produce transplantation immunity and can also produce transplantation tolerance They do not however elicit the Simonsen phenomenon and are therefore not immunologically competent cells

VOISIN And what about epidermal cells ?

MEDAWAR We have not tried using epidermal cells to produce tolerance

BERRIAN Again with regard to the ability of the extracts to bring about tolerance, I think that until you can show that the extracts pre-

pared by any method have all of the complex of factors present when immunization is brought about by living cells you really have no method of testing to see whether cell free material will fail to induce tolerance because of some other properties it may lack I am of course referring only to « T-antigens » and to homologous systems.

WOODRUFF I think there is a way in which one can investigate the question of whether cell free extracts fail to produce tolerance simply because, not being cells they do not survive Give your cell-free extract to the newborn daily or several times a day for a period till you are past the time when tolerance can be produced and *then* give your living cells. It is another of the things we have got on our list but have not yet got round to doing

Prolonged survival of skin homografts in adults (*)

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There now exist two procedures which confer *tolerance* towards a homograft Medawar[1] and his school had the great merit of pointing out, for the first time, that intolerance was no longer an insuperable obstacle. They injected to mice a few days before birth or within the few hours that followed it, living spleen cells from adult mice of another strain and so made these baby mice tolerant to skin homografts from the strain that had supplied the spleen cells.

Main and Prehn[7] then showed that mice X irradiated with a lethal dose and kept alive with intravenous injection of bone marrow from animals of another strain tolerated skin homografts from all the mice from this strain.

Both methods, as such did not seem directly applicable to man, a fact however which did not detract from the importance of these discoveries.

On one hand Medawar noticed soon afterwards, that this induced tolerance was not irreversible and that after injection to these tolerant mice of lymph nodes cells from mice of their own strain there occurs a complete breakdown of the skin homografts they had been tolerating for months.

Trentin[14] used the same procedure in animals made tolerant by lethal doses of X irradiations and injections of homologous bone marrow cells Not only did the animals reject the skin homografts they had tolerated for a long period but they died from progressive anemia induced by the destruction of homologous bone marrow cells that had hitherto saved their lives.

(*) This work has been supported by grants of the « Fonds National de la Recherche Scientifique » and the « Fonds de la Recherche Scientifique Médicale »

On the other hand Billingham and Brent[3] soon drew attention to the danger sometimes fatal of intravenous injection of living spleen cells from certain mice to other mice *genetically too different.*

And finally after Dempster's[4] and Simonsen's[10] suggestions about renal grafts, Simonsen stressed another danger just as great that was afterwards confirmed by other research workers, i.e. living homologous cells that confer tolerance live and multiply in the organism of the receptor if the immunological system of the latter has not reached maturity (as in the experiments of Medawar) or if X irradiation with a lethal dose has paralysed this system. And Simonsen[11] showed by apparently irrefutable experiments, that some animals made tolerant by Medawar's technique, died through a « graft versus host reaction » the homologous grafted cells producing antibodies against the antigens of the host.

Trentin[15] noted similar deaths of X irradiated animals injected with bone marrow *after progressive deterioration of their general health* which he ascribes to the same mechanism and calls « homologous disease ».

All these findings suggested new difficulties, and research workers set about to determine the nature of the antigen or antigens responsible for intolerance to homografts.

To-day we would like to summarize some of our observations made during work that we have been doing for several years and of which we have not yet published the results because at first sight we had the impression that our contribution did not add something really original to what others had already observed. The object of our experiments was to investigate some of the still mysterious aspects which surround the phenomenon of *prolonged survival of skin homografts in the adult* receptor

This phenomenon has generally been considered to be entirely different from that of acquired tolerance and some have tried to assimilate it to the well known *enhancement*[14] phenomenon.

We are not so convinced of this fundamental difference between problems of induced tolerance and those related to prolonged survival of homografts *in the adult. It might only mean quantitative differences* and this problem would remain full of interest providing that one deals with undoubted prolonged survivals and the phenomenon be considered *as a whole without neglecting* any detail in the host both from the general and local viewpoints.

We shall perhaps ask many question and not be able to give as many answers. Loyal comparison and *discussion of results* even para

doxically opposed, of several research workers is undoubtedly the best way of working out the solution to a problem

Finally we shall give some first results of our present experiments

A. — Preparation of receptor animals by previous intravenous injection of epidermal cells from the future skin donor.

I — *Material and techniques*

This part of our work somehow reminds one of the papers published by Billingham and Sparrow[4] who after injection of epidermal cells, noted a temporary but significant survival of homografts from the cell donor In order to keep as close as possible to the conditions realized in the production of acquired tolerance, we only used the intravenous route. Billingham had insisted already on the ineffectiveness of the peritoneal route and on production of accelerated breakdown after intra dermic injection.

The rabbits, all males, that we used were chosen so as to realize a real genetic disparity The donors were albinos rabbits and the receptors grey rabbits. Weight varied between 2 5 and 3.5 kg From three to five days before the graft, the receptor received a single very slow injection of epidermal cells from the future skin donor

Preparation of epidermal cells small fragments of skin taken from the ears and the abdominal wall were submitted to the action of DIFCO trypsin at 1/250 in PBS isotonic sterile solution (*) The epidermal sheets are kept in sterile PBS solution The deep layer is delicately scraped and the malpighian cells dissociated in sterile PBS are centrifugated at 1500 rpm during five minutes. The supernatant is discarded The cells are collected into 3 cc of PBS and suitably dissociated through agitation They are counted by means of a hemocytometer

These details have some importance and Billingham and Sparrow who did their intravenous injections in two steps, insisted on the necessity to get a result, of adding to the second injection part of their crude suspensions of cells in citrate solution We shall come back to this point later The number of injected cells invariably amounts to twenty millions.

Grafts are performed according to Medawar's technique the rabbits being anesthetized with veterinary nembutal (30 mg per kg). Two autografts and four homografts are placed on a large denuded area all is covered with tulle gras and protected with plaster The grafts are

(*) PBS. Formula 0 137 NaCl M 0 0026 KCL M 0 0081 Na_2 HPO M 0.00147 KH PO M 0,0009 CaCl M and 0,00048 MgCl $6H_2O$ M

taken from the ear and their survival is observed every three days. The aspect colour and consistence of the grafts are carefully noted. Histological examinations are done at various phases of the evolution.

At the same time, the host is observed as closely as possible weight blood count protein content and albumin-globulin ratios and for some the cholesterol dosage. Paper electrophoresis of the serum is carried out with a personal technique *

Control rabbits receive the same dose of epidermal cells, but no grafts. Others had no injections, but are submitted to the same analyses.

The average survival period of homografts in our untreated rabbits is 10 days with a margin of ± 1 5 according to the generally accepted average.

Results

The first fact to be noted is that intravenous injection of epidermal cells is not without risks. Some rabbits die in a few seconds and show signs very similar to those of peptonic shock. These accidents depend on the speed of the injection and on the lack of the washing of the cells. Injection of supernatant is particularly toxic it contains many cellular debris.

Evolution of injected and grafted rabbits

First it should be noted that during the first two weeks following the grafts, all rabbits lose weight although feeding normally This is not true for the control rabbits who very often put on weight as do most of the normal animals receiving laboratory diet This first point being established the subsequent evolution in our animals compelled us to divide them into three types

Type I These first undergo a moderate loss of weight then rapidly come back to their initial weight Survival of the homografts varies from 16 to 35 days (average ~8 2 days)

Type II The period during which the animals lose weight is a little longer and whereas survival of the homografts is shorter (average 18.2 days), these animals develop dermatosis. Their general health improves, and they survive

Type III These animals keep on losing weight in spite of practically normal feeding and die between 25 and 44 days after their grafts while the homografts survive

Table I illustrates the usual evolution in this instance, of ten survivors of a group of 14 animals of which four died immediately following intravenous injection of epidermal cells

TABLE I

Number	Injected dose	Survival.
L. 96	20 millions	35 days
L. 97 –	20 »	39 »
L. 98	20 »	25 »
L. 99 –	20 »	27 »
L. 132	20 »	44 »
L. 206	20 »	16 »
L. 209	20 »	35 »
L. 210 D	20 »	21 »
L. 211 D	20 »	18 »
L. 213 D	20 »	16 »

animals which died. D = Dermatosis.

Blood count The three groups noted above may also be distinguished by the different evolution of their blood alterations. Animals of type III develop increasing anemia with leucopenia and fatal evolution. The two other types also have a temporary but moderate fall in their red blood cell count, somewhat more pronounced in rabbits with dermatosis. In these two types, however there is no significant modification of the leucocytes.

Proteins The total protein content as well as the albumin-globulin ratio (determined by chemical analysis and by paper electrophoresis) reveals only slight modifications in animals with dermatosis (type II). Animals of type I while having a longer survival of their grafts, behave in a very similar way In type III on the contrary disturbances are very important the albumin globulin ratio is inverted Electrophoresis confirms the drop in albumin content with a considerable increase of all globulins and especially γ-globulins

Blood cholesterol It undergoes little change in other types but progressively increases until death in rabbits belonging to type III

Evolution of the homografts and of the bed of the grafts

Intravenous injection of epidermal cells of the donor prolongs survival of the homografts in the receptors in all instances, without exception. This sometimes moderate survival may reach and exceed 35 days. In rabbits that die owing to progressive alteration of their general state the graft remains alive till the day the animal dies (44 days in one of them). Only homografts from the donor of the epidermal cells

survive for a longer period this is an individual specificity But if the grafts only have a temporary survival which is not to be neglected however they never undergo the characteristic breakdown of untolerated grafts. For some days the homografts appear exactly as an autograft *they then undergo progressive atrophy* a kind of more or less rapid senescence and are slowly resorbed and replaced by tissue of the host. During the first days, the homograft produces a ringed out growth of epithelial cells. In some cases it is very difficult to say whether small traces of homografts do not remain indefinitely or at least for a long time. We give, as limit of survival the macroscopic appearance of the very first signs of alteration of the graft.

The evolution of the bed of the graft is totally different from what it is in a normal rabbit In the latter the bed is invaded by epithelial growth of the host with cicatricial retraction giving a practically linear scar In all our pre-treated rabbits this retraction is much less pronounced

Another curious phenomenon observed in our animals with dermatosis is the appearance of long white hairs, just at the limit of the *homograft giving finally the aspect of a successful graft of a white* rabbit perfectly tolerated by the grey receptor

It is known that skin homografts of brown or black mice tolerated by a receptor may lose their pigments and finally appear as grafts of white mice[17] But in our rabbits grafts always taken from the ear would never grow long white hairs even if they were autografts It is even a mean of identifying short haired grafts transplanted to a long haired area. In our experiments, although our homografts have a prolonged survival they seldom grow hair and if they do the hairs disappear rapidly Without doubt this is a characteristic difference with grafts transplanted on completely tolerant animals

But how to explain the appearance of large tufts of white fur which grow exclusively near the homografts If this phenomenon occurs in all our rabbits with dermatosis we did observe it once in a rabbit that had no single sign of dermatosis.

We pointed out earlier the enormous toxic effect of the supernatant of our preparation of epidermal cells Out of six rabbits receiving an intravenous injection of the supernatant *five died immediately* The sole survivor contrasting with what is constantly observed after intravenous injection of epidermal cells developed a violent transplantation immunity reaction

Before discussing our experimental observations we had to know if these facts were a *specific response to the preparation of these animals* by an intravenous injection of epidermal cells of the future donor

We had also to eliminate possible bacterial contamination difficult to avoid with epidermal cells. In spite of negative bacteriological controls, we could not guarantee perfect sterility of all our injected material

For these various reasons, we repeated the whole of our experiments this time using spleen cells.

B — Preparation of receptor rabbits to homografts with previous intravenous injection of spleen cells from the future donor.

These experiments were carried out according to the same plan as the former

Spleen cells were prepared by the standard technique of Medawar The spleens are collected with the careful aseptic precautions, cut into small pieces, and passed through a sterile fine meshed-stainless steel sieve. Centrifugation resuspension of the cells in isotonic liquid (PBS) are carried out with all precaution of bacteriologic asepsia Counting is done as for epidermal cells

In order to get results somewhat comparable to our previous experiments, we have to inject a much larger number of spleen cells. Fortunately a very slow intravenous injection of spleen cells is much better tolerated and we have been able to inject, without any disorder other than slight polypnea 700 to 820 millions cells.

Table II shows the survival of homografts.

TABLE II

Number	*Injected dose*	*Survival.*
L 85	710 millions	16 days
L 86	» »	28 »
L 87	» »	34 »
L 88	820 »	24 »
L 89	»	17 »
L 118 D	» »	17 »
L 119 –	»	18 »
L 123 D	» »	35 »
L 65	800 »	38 »

animals which died at 29 and 85 days respectively
D = dermatosis.

With this amount of cells, survival of homografts is rather similar to what we obtained with epidermal cells Two facts however must be mentioned *first* while intravenous injection of spleen cells, if done slowly results in no immediate death two animals out of ten

died one after 29 the other after 55 days although the survival of the grafts was only 24 and 18 days respectively This is in contrast to what we observed for epidermal cells, where rabbits died while their grafts were still living

The *second* rather unexpected point is the appearance in two other rabbits injected with spleen cells of the same dermatosis noted with epidermal cells.

As to evolution of the general health of rabbits injected with spleen cells we find with but little changes the three types of the previous series

Type I shows no appreciable modification

Type II after a slight loss of weight during the fifteen days following grafting the weight returns to normal. The blood count undergoes very moderate and transitory changes. Total protein contents vary but slightly while albumins markedly decrease and globulins increase Return to normal takes from one to four months.

Type III shows progressive and important modifications. The animal loses weight until it dies. At the same time there is progressive anemia although less severe than with epidermal cells. Total proteins do not vary very much but there is an inversion in the albumin-globulin ratio the albumins decreasing as the globulins increase to high levels. Paper electrophoresis shows an increase of all the globulins but principally of the γ-globulins.

Total cholesterol increased slowly from 1 1 gr at the time of the graft to 2 gr shortly before death

Comments and Discussions.

Whether we pretreat with epidermal cells or with spleen cells in both cases we obtain varying survival periods of the grafts and a difference in the general state of health of the hosts which brought us to group them into three types.

Let us first consider the evolution of the general health of the animals, and look afterwards at the problems related to survival of the grafts

A first observation is that control rabbits receiving only a single intravenous injection of epidermal or spleen cells at the doses fixed above undergo practically no or very little alteration of their general health

On the contrary the general health of all rabbits which were grafted afterwards, is more or less *modified sometimes* until death Many

elements are to be considered to the possible effects of intravenous injection of a rather high number of homologous cells, should be added the anesthetic and the operation required for preparing the bed of the grafts, and the actual grafting the wearing of a plaster cast (for about 15 days) and the eventual reactions to the homografts.

Type I slight loss of weight is observed with few biological temporary modifications then the animal rapidly regains weight and behaves practically like the control animals. Here, we would like to make a comment to which we shall refer later *rabbits of this type do not present the shortest survival of their homografts* (we have found for this type, 24 35 and 38 days)

Type II the biological modifications are very similar to the preceeding ones perhaps a little more pronounced and lasting a little longer The main characteristic of this group is the appearance of dermatosis and long white hairs growing from the bed of the homografts. We would like to recall that the disturbance in the protein balance, although transitory seems more important after injection of spleen cells.

A. — *Dermatosis*

Towards the 21st day we observed mainly on the flanks of the rabbits, wide zones where fur grew scarcer and thinner Under the microscope, skin from the affected area showed a somewhat atrophic aspect the malpighian layer being reduced to one or two layers of cells.

So as to avoid any error of interpretation Professor Albert applied to his ever obliging Colleagues of the National Institute for Veterinary Research in Cureghem, and we would like to express our thanks to Professor Willems who examined our rabbits and certified that they were suffering from no known dermatosis, either infectious or parasitary

Appearance of this very special dermatosis in rabbits used for experimental homografts, led us to compare our work with that of Voisin [14] who concluded that there is a possible auto-sensitization

In the beginning (i.e. during the period we prepared rabbits with epidermal cells) we were especially impressed by the similar traits. We saw differences later which we would like to stress here.

First we must point out that our methods for preparing receptor animals differed widely Voisin gives three or four intramuscular injections of total ground skin in normal saline emulsified with regular Freund's adjuvants (with or without mycobacteria) This emulsion is probably slowly resorbed With this preparation which possibly con

died one after 29 the other after 55 days although the survival of the grafts was only 24 and 18 days respectively This is in contrast to what we observed for epidermal cells where rabbits died while their grafts were still living.

The *second* rather unexpected point, is the appearance in two *other rabbits injected* with spleen cells, of the same dermatosis noted with epidermal cells.

As to evolution of the general health of rabbits injected with spleen cells we find with but little changes, the three types of the previous series

Type I shows no appreciable modification

Type II after a *slight loss of weight during the fifteen days follow* ing grafting the weight returns to normal The blood count undergoes very moderate and transitory changes. Total protein contents vary but slightly while albumins markedly decrease and globulins increase. Return to normal takes from one to four months

Type III shows progressive and important modifications. The animal loses weight until it dies At the same time there is *progressive* anemia although less severe than with epidermal cells. Total proteins do not vary very much but there is an inversion in the albumin-glo- *bulin ratio the albumins decreasing as the globulins increase to high* levels. Paper electrophoresis shows an increase of all the globulins but principally of the γ-globulins

Total cholesterol increased slowly from 1 1 gr at the time of the graft to 2 gr shortly before death.

Comments and Discussions

Whether we pretreat with epidermal cells or with spleen cells in both cases we obtain varying survival periods of the grafts, and a diffe- rence in the general state of health of the hosts which brought us to group them into three types.

Let us first consider the evolution of *the general health of the ani* mals and look afterwards at the problems related to survival of the grafts.

A first observation is that control rabbits receiving only a single intravenous injection of epidermal or spleen cells at the doses fixed above undergo practically no or very little alteration of their general health

On the contrary the general health of all rabbits which were graft ed afterwards, is more or less modified sometimes until death Many

elements are to be considered to the possible effects of intravenous injection of a rather high number of homologous cells, should be added the anesthetic and the operation required for preparing the bed of the grafts, and the actual grafting the wearing of a plaster cast (for about 15 days) and the eventual reactions to the homografts.

Type I slight loss of weight is observed with few biological temporary modifications then the animal rapidly regains weight and beha ves practically like the control animals. Here, we would like to make a comment to which we shall refer later *rabbits of this type do not present the shortest survival of their homografts* (we have found for this type 24 35 and 38 days)

Type II the biological modifications are very similar to the pre ceeding ones perhaps a little more pronounced and lasting a little longer The main characteristic of this group is the appearance of dermatosis and long white hairs growing from the bed of the homografts. We would like to recall that the disturbance in the protein balance, although transitory seems more important after injection of spleen cells.

A — *Dermatosis*

Towards the 21st day we observed mainly on the flanks of the rabbits wide zones where fur grew scarcer and thinner Under the microscope, skin from the affected area showed a somewhat atrophic aspect the malpighian layer being reduced to one or two layers of cells.

So as to avoid any error of interpretation Professor Albert applied to his ever obliging Colleagues of the National Institute for Veterinary Research in Cureghem and we would like to express our thanks to Professor Willems who examined our rabbits and certified that they were suffering from no known dermatosis, either infectious or para sitary

Appearance of this very special dermatosis in rabbits used for expe rimental homografts, led us to compare our work with that of Voisin [14] who concluded that there is a possible auto-sensitization

In the beginning (i.e during the period we prepared rabbits with epidermal cells) we were especially impressed by the similar traits. We saw differences later which we would like to stress here

First we must point out that our methods for preparing receptor animals differed widely Voisin gives three or four intramuscular injec tions of total ground skin in normal saline emulsified with regular Freund's adjuvants (with or without mycobacteria). This emulsion is probably slowly resorbed With this preparation which possibly con

tains living cells but also certainly and perhaps mainly cellular debris, Voisin observes an accelerated breakdown of skin homografts from the rabbit which supplied the ground skin.

We prepare our animals with a *single intravenous* injection of epidermal cells, and we thus obtain prolonged survival of the homografts from the donor rabbit. *However we observe identical dermatosis.*

Thus, we are able to conclude, like Voisin that the adjuvant substances of Freund are out of question. But may we conclude, as he does, that in our cases there is very likely an auto-sensitization?

A first finding left us thoughtful we have observed exactly the same dermatoses in rabbits prepared with an intravenous injection of spleen cells. It could be admitted at least in our case with spleen cells, that auto-sensitization would mainly be induced by the homograft itself But then why do not all grafted rabbits develop dermatosis?

There is a last difference, i.e. while Voisin's autografts behave in rather the same way as homografts, our autografts took in quite a normal way the first being contemporaries and controls of our homografts, the second being done in the full period of dermatosis, the grafts having been taken from healthy areas as well as from dermatosic ones.

In spite of these differences, we have no right to neglect this phenomenon, the solution of which might bring some useful contribution to the study of grafts, and perhaps should be studied more closely This is one of the points we should like to discuss during our colloquium.

Let us mention that injections of B⁴ to rabbits with dermatosis in no way modified the evolution of their dermatosis.

B — *Appearance of long white hairs in grey rabbits on the bed of old homografts*

We shall not refer at length on this curious occurrence we have observed in all dermatosic rabbits prepared with epidermal cells. We have never seen it in dermatosic rabbits treated with spleen cells In one single rabbit, however injected with epidermal cells this phenomenon occurs in a more discreet way without dermatosis. These long white hairs have always grown on temporarily tolerated homografts progressively absorbed by the host.

The appearance of these *white hairs is thus not necessarily related to* the dermatosis but it seems clearly in relation with intravenous injection of *epidermal cells* combined with homografts. It never occurs in other parts of the graft beds. However one of the grey rabbits with dermatosis injected with epidermal cells, is now developing 105 days after the first grafts zones of white fur *along the edge of the ears*.

All this may be a very accessory phenomenon with which we shall deal during the discussions. In biology one should never at first sight, belittle an observed fact just because there is no satisfactory explanation.

Type III This last type, rather important, represents an average of 30 % of rabbits pretreated with epidermal cells and 20 % of those who received spleen cells.

A graph shows the evolution of their weight curve, their anemia of modifications in their protein and cholesterol rates. All these animals died but their autopsy never yielded any conclusive revelation The most striking element was an hypertrophy of the fasciculated layer of their adrenals which sometimes doubles its normal weight, phenomenon without any specific significance. So far neither macroscopic nor microscopic examinations revealed appreciable alterations in the lymphatic system or in the spleen

By careful histological examinations of the lungs we found, in one single case pretreated with epidermal cells some whitish nodes of living cells with an epithelioid aspect. We have not been able to identify it with certainty so far Discovery of injected epidermal cells is rather difficult but from a doctrinal viewpoint the question is important we shall consider it again when dealing with prolonged survival of the homografts.

So far we have been unable to voice anything definite either on the mechanism of relative alteration in the general health of animals belonging to types I II or on the cause of the deep and lasting disturbances that bring animals of type III to their death. Should survival of homologous cells be incriminated or their destruction? Whatever the explanation may be, the homografts seem to play perhaps a secondary but determinant role.

Prolonged survival of homografts

The careful study of this survival and concomitant phenomena constitute an interesting part of the whole problem of tolerance and intolerance.

How do homografts behave in animals pretreated with intravenous injections of epidermal cells? Experiments with spleen cells used as controls gave similar results. We shall not refer to them here again.

All these experiments have been carried out on rabbits In mice, we found hypersensitivity to these intravenous injections, with a very high mortality rate. The survivors showed prolonged homograft survival as did the rabbits.

The evolution of the homografts is however slightly different in rabbits and in mice. In the first, the homograft behaves as an autograft except that it becomes progressively thinner decreases in size and seems finally incorporated into the tissue of the host This is very similar to what H W Toolan[18] described in her animals treated with cortisone We noted the hyalinisation in the ground substance and a very late appearance of a moderate leucocyte invasion particularly of lymphocytes. Is this scarcity the cause or the consequence of the slow resorption? This question is as yet unsolved.

In mice after a few days, the superficial layers of epiderm fall off leaving beneath a living graft with two or three layers of malpighian cells. Afterwards the involution is similar to that described in the rabbit One might wonder although we do not wish to assert anything yet whether in some cases part of the homograft does not persist for a long time if not indefinitely

In both animals, if some hair grows on the homograft they always disappear and never return.

In all rabbits prepared with a single intravenous injection of epidermal cells, we observe significant survival of skin homografts of the same donor This survival of 15 days at least may reach 31 35 and even 40 days or more and appears as an individual specificity As Medawar showed that 3 to 5 days are sufficient to induce a transplantation immunity all our animals were grafted 3 to 5 days after injection. How does this injection of living homologous cells act and how is it that they do not produce transplantation immunity? They prolong not only the survival of a homograft from the same donor but they prevent also a second set reaction of a second graft.

Until further information to get these expected results one has to use living cells. May we recall the entirely opposite results induced by epidermal cells and their supernatant. The latter contains numerous cellular debris which are highly toxic and provoke an accelerated break down of the homografts.

But do these living homologous injected cells act as such multiplying in the organism of the host or do disintegration of these cells and liberation of their antigens partly neutralize the antibodies that the host might develop towards the homografts?

A first question should be solved and we are working on this problem. How can homologous cells injected intravenously to a normal receptor live and eventually multiply without being destroyed and eliminated within a few days? It might be a dosage effect which overwhelms the normal immunity defences of the host But to support this we have to prove the presence of these cells their vitality and even

their proliferation If this theory should prove correct the active part of these living cells in survival of homografts could be suspected as well as their influence on the fate of the host This would suggest « graft versus host reaction » but why does this « graft versus host reaction » not appear in the controls injected with the same number of homologous cells, but not grafted ? On the other hand are epidermal cells immunologically competent i.e. are they able to produce antibodies ?

Whatever the response may be what is the part played by the cells or by their metabolites in this process ?

As all our rabbits undergo at least a slight alteration of their general health one might attribute the survival of the grafts to a lack of defence of the organism But in our mind the problem is not so simple and prolongation of homograft survival is far from being proportionate to the alteration of the host We have survival periods in practically normal animals which are sometimes shorter in animals slowly deteriorating

Could we think of a cortisone effect as in the experiments of H W Toolan hypertrophy of the adrenals in animals that die and progressive increase in their cholesterol contents could appear to be in favor of this theory But our homografts survive as long if not longer in animals with non varying cholesterol contents. One could perhaps suggest experiments on normal animals with dosage of the cortico-steroids or on animals deprived of their adrenals. But many animals are unsuited for such experiments, and valid results would require very large statistics

Do we deal here with an immunological paralysis in the sense of Felton ? This interpretation is not to be discarded We would mention however that we have tried to paralyze the immunitary system by various procedures without modification of the survival of our homografts. This will be the subject of another paper

We were thus so far faced with problems still unsolved and we had to take into account the severe risks for the host of the intravenous injection of living cells. Simonsen suggested the use of immunological immature embryo cells To avoid these disasters we deliberately tried to approach the problem by another way

Introduction

The hypothesis according to which tissular groups giving rise to incompatibility reactions similar to those of blood groups would be responsible for transplantation immunity has been examined in 1947 by Medawar [1] Since then the study of blood group substances became of increasing interest in the field of immuno-chemistry The agglu

tination of red blood cells has been inhibited by adding blood group substances in vitro as well as in vivo (*) And so we wondered whether it was not worth while to reconsider the question on the level of homografts. *Our first intention was to see whether organ extracts containing group substances, would modify the normal course of disintegration* reaction of the homograft in mice.

Technique

We used organ extracts prepared according *to the technique of* Friedenreich and Hartmann[4] for inhibition tests on hemagglutination These extracts mixed with their volume of water are kept for ten minutes in boiling water They are afterwards evaporated at normal temperature. The dry extract is then dissolved in a suitable quantity of sterile normal saline.

So far we tested principally stomach or spleen extracts. Others are now *being tested Until now* injection and graft are performed on the same day We tried the intravenous *the intra peritoneal and the intra* muscular route. For the homografts, we use Medawar's technique Receptors are champagne coloured mice donors are C[57] black

Results

1 The average survival period of skin homografts in our mice previously determined is 10.5 days ± 0.5

2. Isologous extracts have given no survival prolongation of the skin homografts.

3. As to homologous extracts, results are listed in Table III

TABLE III

Routes.	*Extracts*	*Contents (Dry extract)*	*Survivals (days)*	*Average*
Intravenous	Stomach	4.4 mgr	16 to 20	18
	Spleen	3.5	16 to 18	17
Intraperitoneal	Stomach	5	22 to 24	23
	Spleen	4	16 to 18	17
Intramuscular	Stomach	6	10 to 16	13
	Spleen	7	16 to 18	17

(*) For further information, see KABAT *Blood Group Substance* 1 vol Acad Press, New York.

Comments

A first point worth while mentioning is the absolute innocuity of the extracts at the dosages mentioned. We did not have the slightest incident. Intravenous injection is of course, slowly done. Some animals have been watched for a long time they showed no alteration of their general health and we did not see any deaths similar to those described in rabbits after injection of epidermal or spleen cells.

As for homografts, they behave during their first survival period nearly like autografts Towards the 14th day the superficial layer of the epiderm scales off leaving beneath two or three layers of malpighian cells. Sometimes, a few hairs grow on about the 18th day After the 20th starts a slow and progressive resorption In some instances, however we found grafts to be in very good condition towards the 40th day

It is still to early to discuss the mechanism of prolonged homograft survival by this procedure but there may be a possible specific inhibition of disintegration by the mucoids present in our extracts.

These experiments are now in progress on a large scale. Baby mice have been injected at birth and we shall soon be able to control the results. We still have to study the influence of doses, time and number of injections, we are also endeavouring to determine the composition of the extracts so as to ascertain which element, or elements are active. Our first results are undoubtedly encouraging and we hope to bring to the Colloquium the remainder of the observations we shall make during the next few weeks.

It is not without interest to record, as from now, that it is possible to obtain prolonged survival of homografts in adults, by means of non living heat stable substances from the future donor of the homografts

ADDENDUM.

Now we can already give some more comments about the activity of our extracts. Up till now they do not seem to give any sign of the specific activity of a transplantation antigen.

The absence of any T antigenic activity whatsoever has been supplied by the two methods described already by Medawar i.e.

1 Induction of tolerance.

2 Antigenic activity tests of transplantation immunity in the adult animal.

1 Our experiments have shown that of 40 new born mice injected at birth and alive at the time of grafting, none showed the slightest prolongation of survival of their skin homografts All animals disin

tegrated their grafts within normal delays about the 10th day With one single injection these extracts do not appear to induce tolerance. A further series of tests will have to be carried out with repeated injections

2 Antigenic activity test of our substances in the adult animal. We have never observed phenomenae of transplantation immunity with graft destruction on the 6th day

These two series of experiments have proved that we are not dealing with a specific reaction due to an antigen which would play a part in transplantation immunity phenomenae.

Owing to the universal difficulty in obtaining *considerable quantities* of mice for such experiments we turned to rabbits, a little against our will. It is known and other experiments confirm this that rabbits give little homogenous response to various antigenic stimuli as far as immunology studies are concerned *Moreover* very wide genetic diversity complicates considerably the problem

Out of 12 rabbits injected with gastric extracts 6 had a homograft survival of 14 to 18 days 2 others disintegrated within normal delays while the 4 last rabbits discarded their grafts on the 12th day

TABLE.

			Survival.
1	L.	229	18th day
2.	L.	233	12th »
3.	L.	234	16th »
4.	L	237	12th »
5.	L.	238	14th »
6.	L.	239	12th
7	L.	240	10th
8.	L.	263	10th »
9.	L.	257	12th »
10	D	143	17th »
11	L.	251	13th
12	L	271	18th

The analysis of gastric extracts by means of paper electrophoresis, under well set conditions shows that they contain a non negligeable proportion of mucopolysaccharides Thinking that the latter might after all perhaps play an important role in skin homograft survival prolongation in our animals we wondered whether injecting our animals with sufficiently large quantities would not ensure better survival of the graft using multiple injections

As in the rabbit repeated removal of the mucopolysaccharides from the gastric lining is difficult we tried to collect these substances *in toto* from the saliva To do so it is collected under general anaesthesia *after intravenous injection of pilocarpine* chlorhydrate The volume of saliva collected from rabbits weighing 3 kg varies between 9 to 15 cc The saliva is treated in the same way as the gastric extracts

We have tested the recipient by means of homografts from the donor of salivary extracts. Our experiments are under way

So far the injection seems devoid of all danger even by the venous route. Before and during all the time of evolution of the homografts blood compatibilities are tested in dextran.

Starting with animals with a definite agglutinin titre in dextran solution we tested and observed *in vitro* inhibition of the hemagglutination by addition of salivary extracts. But if extracts from the future donor are injected intravenously *in vivo*, the titre decreases in some cases, and is hardly modified in others.

Our results, so far still fragmentary allow us the first transitory conclusions

1 When the skin homograft is done, hemagglutinin titre in dextran solution appears or increases at the moment of disintegration of the graft.

2 In some cases one observes a decrease in hemagglutination titre after multiple injections of salivary extracts. During this period a homograft shows a prolonged survival When the titre of hemagglutinins is not modified even after several injections, the homograft will disintegrate within normal delays and at that time, hemagglutinin titre rises

It would be premature to draw a conclusion from these findings. But so far we have noted that at the time of disintegration of the homografts in all our rabbits, with or without injection of our extracts hemagglutinins appear or increase their titres

We think it would be interesting to study more deeply the relations that may exist between apparition of hemagglutinins or their disappearance and the evolution of homografts.

Considering the marked difference of evolution of homografts in mice and rabbits under influence of our extracts the comparative study of that parallelism in these two animals might shed a little more light on these problems.

This is what we are trying now to establish

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DISCUSSION

VOISIN Since Professor Albert asked for my opinion I will make 3 comments About the question of dermatosis, I will first remind you of our early work with Dr Maurer in which we injected homogenates from prospective skin donors mixed with Freund's adjuvants into prospective recipients. We then transplanted to these injected rabbits skin homografts from the same donors. These homografts exhibited second set reactions. Establishing this point was the only purpose of our experiments. About two months later we noticed on the animals which had received skin homogenates and homografts a peculiar kind of dermatosis consisting of loss of hair and thinning of the epithelium Furthermore, autografts performed on these rabbits did not enjoy a perfect take as they should have done but exhibited certain abnormalities around the eighth day Now two animals had received the skin homogenate but no graft, and these did not exhibit dermatosis. In our experience the only animals in the experiment which showed a dermatosis were those which had received both the homogenate mixed with Freund's adjuvants (with or without mycobacteria) and a subsequent graft The veterinarians from the National Veterinary School of Maison Alfort told us that they had never seen such a dermatosis We then tried to

investigate this question and go further In order to have a fairly large series of rabbits, we started an experiment on 50 rabbits at the Veterinary School After 2 weeks the animals were swept off by an epidemic Being at that time engaged in other experiments we did not go further But during the following years we noticed a few facts in connection with this. Several rabbits which had received one or two skin homografts or heterografts, and several guinea pigs which had received skin homografts showed a dermatosis, more limited but of the same kind If we consider now Drs. Albert and Lejeune's experiments, we see that the minimum requirement for the dermatosis to appear is the injection of epidermal or spleen cells followed by a homograft. All this, without furnishing an explanation suggests that the process is due to some kind of skin specific sensitization which would occur independently of the individual specific sensitization leading to homograft rejection Nevertheless, I must point to the fact that I have observed two rabbits showing a dermatosis having the same appearance after having received several injections of kidney homogenate mixed with Freund's adjuvants. That is the reason why I do not feel as confident today about the specificity of these skin lesions as I was a few years ago The problem is still open

My second comment concerns the increase of serum gammaglobulin and decrease of serum albumin in Drs. Albert and Lejeune's rabbits whose health was impaired. Two years ago working in Dr Hahn's laboratory in Nashville Ten I tried to see whether or not protein depletion in dogs would allow homografts to live longer by impairing the production of antibodies or by weakening the immunological mechanism in some other way Dr Hahn used to deplete his dogs of proteins by repeated bleeding associated with iron injections and low protein diet. It was quite striking to observe the modifications of serum proteins there was a sharp decrease of the albumin level and a paradoxical increase of the gamma globulin level

The third comment will concern what Dr Lejeune told us about the fact that their mucoid preparations, instead of immunizing the recipient against the » mucoid donor » did in fact allow the grafts to live longer This is absolutely fascinating These preparations might be comparable to Kandutsch's extract Since isologous mucoids have no effect and only homologous mucoids coming from the donor's strain it is perfectly possible that Dr Lejeune has prepared an « enhancing antigen » toward normal tissues. If these facts are confirmed they will prove to be the most important contribution to the symposium and to homograft problems for several years

LEJEUNE Thank you very much Dr Voisin for your kind appreciation Of course just as you suggest we have also thought about an enhancement phenomenon But for the time being as far as normal tissues are concerned there are still too many variations in the interpretation of the term « enhancement » and we have preferred not to raise that special point The problem is still too complex to suggest any conclusive explanation at this stage of our experiments. We think before searching for an interpretation we want to have much more experimental material and particularly comparative studies on the reasons why rabbits and mice react in a very different way

MERRILL I would just like to ask one question. I must reorientate myself in the clinical field again You mentioned that both with your injections of epidermal and spleen cells, your animals showed a decreased albumin-globulin ratio and an elevated cholesterol and they lost weight This would strongly suggest in any other animal that it had glomerulonephritis—a nephrotic syndrome Did you check the urine and kidneys pathologically ?

LEJEUNE We can assure Dr Merrill that the urines have been checked regularly and that they were quite normal Even the level of blood urea was normal

MEDAWAR May I raise two very brief questions on this extremely interesting paper ? The first is that there is something immunologically peculiar about the intravenous route of injection in rabbits and I do think that it is important to establish the occurrence of these phenomena in at least one species other than rabbits. I am referring here particularly to the experiments described by Prof Albert The second point is that I really have no doubt in my mind that the « white hairs » described by Prof Albert are simply a particular manifestation of hair neogenesis first described by Breedis and since worked upon by Billingham and Russell Why it should not have occurred after splenic injections I do not know But it can occur in ordinary wound healing without any grafting at all

ALBERT In answer to the first question we can say that in our experiments the prolonged survival of the homografts after intravenous injection of epidermal cells is not peculiar to rabbits. We tried the same intravenous injections in mice and observed exactly the same reactions. But we had a discouraging number of early deaths when we worked on mice and that is one of the reasons why we continued the greater part of this work on rabbits

About the second question we never attached any great importance to that appearance of large tufts of white hair We have drawn attention to it simply because it happened exclusively in rabbits injected with epidermal cells and on the beds of the homografts. Controls have shown that on similar but not homografted beds this never happened

SIMONSEN As to these peculiar cells which Professor Albert observed in the rabbits injected with epidermal cells I do not purport to know the explanation But I do think it has nothing to do with a graft versus-host reaction which was one of the possibilities you suggested and I must say that the slide you showed from rabbit lung containing these enlarged cells with a light cytoplasm did not impress me as being epidermal cells. I think they looked strikingly like phagocytosing histiocytes. I think they were of mesenchymal origin

ALBERT Yes. There is just one point The fixation was not appropriate for a differentiation of the cells. We always hoped to find these cells again but it is very difficult to find such very small accumulations of cells in the lung of rabbits But what is the reason for the deaths ?

SIMONSEN It never occurred with autologous cells ?

ALBERT No never

MEDAWAR Not even the immediate deaths

ALBERT No

LEJEUNE We injected albino rabbits with 5 million pigmented rabbit cells in order to try to detect them by means of their pigmentation

BRENT I would like to point out that the intravenous injection of small doses of homologous spleen cells in the rabbit has shown that there is not necessarily anything very peculiar about the intravenous route in the rabbit Mitchison Billingham and I have injected 5 million spleen cells and were able to show that these rabbits responded in a perfectly orthodox manner they became immune against skin grafts transplanted to them from the spleen donor The prolongation of graft survival in Prof Albert and Dr Lejeune's experiments is much more likely to have something to do with the high dosage of cells injected Zotikov has recently shown that the rabbit is capable of producing straightforward saline-agglutinating antibodies and I wonder whether they have determined the antibody content of the recipient sera after

injection of either homologous spleen or epidermal cells. It may be that this is just another case of enhancement. A graft-versus-host reaction seems distinctly possible in the case of the spleen cells. I do not know whether you have ever observed lymphoid involution in your rabbits after spleen cell injection. But that kind of reaction is rather too much to ask of epidermal cells. Some kind of enhancement phenomenon seems more likely to me.

OWEN: I would like to ask Dr Lejeune if he has ever found in the serum of an untreated rabbit any normal antibody that will agglutinate the red cells of other rabbits by your test techniques.

LEJEUNE: In our experiments we have never found agglutinin in normal saline, but in dextran solution we found a small titre of agglutinin in normal conditions just before injecting our extracts. This titre was low: about 1/8 or 1/16.

SISKIND: First, with regard to time relations: how soon after your initial injection and how long after your initial injection can you delay grafting and still see this type of delayed death?

Second: Can you substitute for the skin grafts a second injection of epidermal cells and still see delayed deaths?

ALBERT: We have never tried it. We never repeated the injections.

SISKIND: What I had in mind with the second question was the possibility that if the donor cells remain in the recipient after the first injection, possibly a second injection or a graft, eliciting a homograft reaction, would cause some sort of antigen-antibody reaction to take place wherever the donor cells had colonized, with resultant damage to the host itself—something similar to an anaphylactic reaction.

VOISIN: Surely you mean a tuberculin-type shock.

SISKIND: Yes, a tuberculin shock type of reaction.

Diversity of transplantation antigens in the mouse

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INTRODUCTION

The antigens responsible for homograft immunity in the mouse are products of co-dominant genes at many different histocompatibility loci. In theory the least number of distinct substances or specific groups implicated for a particular donor recipient combination is a function of the number of disparate genes involved, and it is evident that the recipient *can* develop immunity against all of their products. One important question however is if all of the antigens *are* involved under the usual circumstances of homograft rejection. Recent lines of evidence point to the fact that each of the histocompatibility genes —or antigens— is not equivalent in transplantation immunity and the present study examines the basis for this diversity.

By appropriate genetic methods it is possible to estimate the number of genes setting donor and recipient strains of mice apart on transplantation. Thus, disparity has been found to range from a single gene setting « co-isogenic resistant » (IR) strains apart[16] to 15 or more genes for more distantly related combinations[1]. Estimates of other strain combinations have fallen between these two extremes[10, 11].

Variations in the force of individual genes have been most convincingly demonstrated by the exchange of skin homografts between IR strains. For example Counce et al[8] have found that grafts trans-

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planted across a single gene incompatibility at the H 3 locus survive almost three times as long as those involving an H 2 allele. Other studies have shown that skin homografts may survive in the face of certain weak genes longer than 100 days whereas they are usually rejected in 9 to 12 days when the incompatibility includes a gene at the H 2 locus [1,3] It is apparent that the duration of primary homograft survival is not necessarily dependent on the cumulative effect of a large number of genes so much as it is on the strength of one or more genes in the combination whose antigens set the pace of the processes leading to rejection.

Faced with these facts, there is provocative evidence of an underlying diversity in the properties of the transplantation antigens, as also perhaps, of their distribution and concentration within tissue cells. This may simply be an expression of the fact that the collection of genes, assembled as a group through the fortuitous involvement of their specific products in transplantation may serve in a variety of unrelated physiologic roles. The determinant sites responsible for iso-antigenicity may be quite trivial incidental features of a number of molecules whose remanent structures are vastly different. These speculations cover an area of relationships of which we are largely ignorant, but which was unapproachable in the absence of means for identifying the individual antigens. *The experiments to be described were aimed primarily at this* immediate problem It will be shown that several antigenic factors have been identified among the mice of four different inbred lines. The transplantation antigens « thumb-printed » by these factors predominate in the immune rejections of skin homografts exchanged between the strains in each of the several donor recipient combinations possible.

Design

The experimental plan hinges first of all on the contingency that different inbred strains of mice share some genes in common just as they differ by others The large number of independent loci controlling transplantation in the mouse provides a high probability that any two different strains (donors) will share one or more genes hence the antigens, that a third strain (recipients) *will lack. When this situation prevails*, a primary exposure of the recipients to the antigens of one donor *strain may* result in an immunity detectable not only by the premature second-set rejection of later skin homografts from the *initial donor* strain but also by homografts from a *second* donor strain.

Involved secondly is primary immunization by two alternative preparations (*a*) a viable spleen cell suspension and (*b*) antigenic extracts of spleen cells, prepared accordingly to the method of Medawar and his associates [4,5] The rationale of this comparison is that certain antigens

present in the former preparation may not survive the process of extraction hence they would not be found in the latter source. This is the result of the inherent instability of these antigens and it will be shown later that the comparison offers important evidence of the diversity of the substances.

The use of four different strains of mice offers a large number of strain combinations (36) for comparing the ability of primary immunization to induce second-set responses 12 test the ability of the recipient to reject later skin homografts from the same donors used for immunization and 24 combinations involve different primary and secondary donor strains Testing in these combinations with both cells and extracts for primary immunization takes in a total of 72 different reactions. For convenience the following notation has been adopted if A, B and C are different strains of mice then A → B → C + denotes a test in which B strain recipients were immunized initially by A strain spleen cells or extracts and C strain skin homografts were rejected by means of a *second set* response when applied to the same B strain hosts a result indicated by the positive symbol (+)

Experiences from conventional serology dictate the principles involved in interpreting results of this kind. The antibodies elicited by most antigens include a host of reactive units having a broad range of specificity The fact that members of such a population can react with a variety of different antigens suggests that confirmatory evidence be sought wherever possible for the relationships defined through antigen antibody reactions. Because of the uncertainty attached to interpretation of results the antigenic relationships are defined, symbolically in terms of antigenic factors. The resulting series of symbols has no material correspondence to the antigens themselves, but properly applied they can be used to indicate the presence or absence of antigens and that is the significance attached to them in the present study [14]

The single reaction and its result A → B → C + can be explained either on the basis of shared antigens or through an immunologic cross-reaction. If A and C share identical antigens, the results should be little different if the primary and secondary donors are reversed thus C → B → A However if the results do not conform on reversal of the test, then there is reason to suspect that the positive result had its basis in cross-reactivity It is the negative result that provides more compelling evidence in these tests and the final translation of the results is based on coincidence of all the data in the battery of results. The negative results (homograft survival) carry the greatest weight for two reasons. First homografts may frequently fail for technical reasons, but they rarely survive in the face of strong immunity unless that immunity is not directed against them Secondly it is an accepted immunologic

doctrine that recipients will not respond against those antigens they share in common with a donor If the B recipient immunized against A antigens, rejects an A graft prematurely but not a C graft, then A and C strains obviously share no antigens that B lacks.

MATERIALS AND METHODS

The four inbred strains of mice used in the design are abbreviated, for simplicity as A B C, and D for the lines A/HeN, C57BL.10/ScB₁N C3H/HeN and C57BL.10/H—2ᵇBsN respectively Each of the donor recipient strain combinations differs by a number of « strong » genes such as those at the H-2 locus with the results that primary skin homografts are uniformly rejected between the 9th and 10th days. However when the recipients in any combination are first injected intra-peritoneally with a dose of 1/4 of the cells expressed from the spleen of any donor i.e. 50-70 million cells, and then grafted 5 days later with skin *homografts from the same donor strain* these virtual second-set homografts are rejected by the 6th day In the absence of preimmunization the test grafts in all combinations save one are found to be fully surviving, and well healed on their beds on the 6th day A small proportion of C strain grafts were found to be affected by primary immunity developing in their D strain hosts on the 6th day in the absence of prior treatment. First-set grafts in this strain combination rarely survive longer than 8 days.

The violent second-set rejection of homografts by the 6th day could also be brought about in 8 of the 12 control combinations when the antigenic extract prepared from 400 to 500 mgm of spleen was injected intraperitoneally 5 days *before* test-grafting Neither *A* nor *B* strain recipients could be immunized with antigenic extracts of C strain spleen *so that later C strain test grafts were prematurely rejected* nor was it possible to immunize A or B strain hosts with D strain antigenic extracts, so that strong second-set responses attended later D strain test grafts.

The technique of skin grafting was identical to that of Billingham and Medawar [1] The criteria for determining the condition of the grafts discussed by Billingham Brent and Medawar [2] was based on the extent of epithelial survival healing to the graft bed, and on the establishment of vascularization However for objectivity only 3 scores are recognized here. In the results each (—) symbol indicates a graft which was fully surviving and showed no signs of rejection by a *second-set type of response* i.e. as evidenced by weak attachment to the graft bed Each (±) symbol indicates a *graft whose condition* was in an intermediate status between those grafts marked (—) and grafts which were fully destroyed or *showed* little surviving epithelium (graded +) or were so weakly

healed on their beds as to obviate the necessity of appraising their epithelial survival

Antigenic extracts Aqueous antigenic extracts were prepared as previously described[3] Spleens were excised from their donors, weighed and expressed through a sieve into buffered normal saline (9 parts 0 15 NaCl 1 part 0.1 M sodium phosphate buffer pH 7.4). The cell suspension was washed once and the sediment resuspended in normal saline, to which 3 volumes of distilled water were then added The swollen cell mass was again spun down and the supernatant discarded. The sediment was then dispersed in water by vigorous pipetting, the volume was reduced to the minimum necessary to provide a manageable suspension. Homogenization was effected by the exposure to a 100 watt 20 Kc. magnetostriction transducer The end point was judged by the viscosity of the solution which is reduced sharply as complete homogenization is approached Usually about 5 minutes of exposure were required to homogenize the swollen cell mass from 5 g of spleen The homogenate was centrifuged at 2500 RCF for 5 minutes to determine the completeness of homogenization. Any sedimented cells were removed at this time and rehomogenized following which the supernatant was made up to 0.15 M with concentrated sodium chloride solution The precipitate was removed by recentrifugation at 2500 RCF for 10 minutes and the remaining supernatant was injected in divided doses into the recipients.

Single experiments involved most conveniently the immunization of six mice from each of three strains with equal doses of an antigenic preparation (either cells or extract). Caution was exercised to select recipients of uniform weight and sex and to insure the injection of equal doses of the antigenic preparation. In this way it was possible to evaluate any marked differences in the response of the various strains to the antigens of the 4th strain. Test grafts were applied to both sides of the chest so that 4 of the 6 animals of each strain received one graft each from donors of the same strain used for initial immunization. These provided a control on the effectiveness of the immunizing preparation Each of these 4 animals and the 2 remaining animals in each group received grafts distributed equally from donors of the remaining two strains not involved for initial immunization or as the recipients. Inspection of the grafts was made on the 6th day and their condition noted at that time.

RESULTS.

The scores of the control grafts are assembled in Table I according to the strain combination. These grafts are of the same strain as that used for immunization The scores are assorted in two classes, for both

cells and extracts, because immunization was effected by batches of materials prepared on different occasions. The potency of the batches differed somewhat as evidenced by the results, and the classes were so arranged that the scores of animals receiving the more potent material appear in columns 1 and 3 the weaker in columns 2 and 4 Each symbol (+ ± —) indicates the condition of a single graft. The experimental strain combinations, involving different primary and secondary donors, are given in Table II

The hemagglutinogens (H-antigens) associated with H-2 alleles of the strains used here have been previously described in terms of a collection of symbols or antigenic factors[11, 12] H-antigens are distinct from T antigens in that the former do not provoke transplantation immunity at least to our present knowledge[4, 5] The relation between the two kinds of antigens will be drawn later in the discussion The symbols listed are those factors present in both donors, but not in the recipient hence they are indicative of the incompatibility anticipated at H-2 involving H antigens.

TABLE I.

Survival of grafts in control combinations at six days

Strain combination.	H-2 Ag factors.	Cells 1	Cells 2	Extracts 3	Extracts 4
A → B → A	CDK	+ +	+ +	+ + + +	+ ±
A → C → A	DE	+ +	+ +	+ + + +	+ +
A → D → A	EK	+ +	+ +	+ + + +	+ +
B → A → B	D^a	+ + + +	+ ±	+ + + +	+ +
B → C → B	D^aF	+ + + + +	+ +	+ + +	+ ± ± +
B → D → B	D^a	+ +	+ +	+ + —	+ + +
C → A → C	—	+ + + +	+	± — —	— — — —
C → B → C	CK	+ + + + +	+	± — — —	— — — —
C → D → C	EK	+ + + +	+	+ +	+ + ± ±
D → A → D	E^d	+ + + + +	+	± — —	
D → B → D	CDE^d	+ + + +	+	+ ±	
D → C → D	DE^dF		+	+ +	±

\+ complete destruction
± partial breakdown
— complete survival

The experimental results (Tables I and II) were translated into the tables of factors (Figures 1 and 2). The experimental reactions defining shared antigenic factors are listed in Table II by pairs and one reaction is simply the reverse of the other Where positive result second set reactivity) were obtained in both directions for example A → B → C

TABLE II.

Survival of grafts in experimental combinations at six days

Strain combination.	H-2 Ag factors	Cells 1.	Cells 2	Extracts 3	Extracts 4
A → B → C	CK	+ + +	+ + +	— — — —	± — — — —
C → B → A	CK	+ + +	+ + +	± — — —	+ + — —
A → B → D	CD	+ + +	+ + +	+ + + +	± — — — —
D → B → A	CD	+ + +	+ + +	+ + + +	+ —
A → C → B	F	+ + +	+ + +	± ± — —	— — — — —
B → C → A	F	+ + + + — —	— — —	± — —	— —
A → C → D	DF	+ + +	+ + +	+ + + +	+ + ± — —
D → C → A	DF	+	+ + +	+ + + +	+ ±
A → D → B	E	+ + +	+ + +	+ + + —	— — — —
B → D → A	E	+ + +	+ + +	+ + + +	— —
A → D → C	EK	+ + +	+ + +	+ + + ±	+ + ± ±
C → D → A	EK	+ + +	+ + +	+ ± ±	+ + — —
B → A → C	—	— —	— — —	± — — —	— —
C → A → B	—	+ + — — —	— — —	— — —	— — — —
B → A → D	—	+ +	— — —	± ± — —	— —
D → A → B	—	+ + — — — —	— — —	— — —	— —
B → C → D	F	+ + + + — —	+ ± —	+ + + +	— —
D → C → B	F	— — — —	— —	+ ± ± ±	— —
B → D → C	E	+ + +	+ + +	+ + + ±	— —
C → D → B	E	+ + ± ±	— — —	— — —	— — —
C → A → D	—	+ + ± ± —	— — —	— — —	— — —
D → A → C	—	± ± — — —	— — —	± — —	— —
C → B → D	C	— — —	— — —	± — —	— — —
D → B → C	C	— — —	— — —	+ + — —	± —

\+ complete destruction.
± partial breakdown.
— complete survival.

C → B → A⁺ a symbol « a » designating an antigenic factor is tentatively assigned in Fig. 1 to the two donor strains (A and C) and the absence of that factor in the recipient strain (B) is noted (—). The symbol ∼ is assigned to the strain not involved in the test indicating that neither the presence nor the absence of the factor is known for that strain.

If positive results are found for the reaction in one direction but not in the other for example, B → C → D D → C → B⁻ and B → D → C⁺ C → D → B⁻ the positive results are tentatively ascribed as immunologic cross-reactions. The presence of a factor is not proved in

STRAIN

A	B	C	D
a	—	a	~
f	~	f	—
b	—	~	b
d	~	—	d
c	c*	—	~
e	e	~	—
—	~	~	g
—	~	h	~
—	i	~	~

FIG 1 — Antigenic factors, cells + extracts

STRAIN

A	B	C	D
f	~	f	—
bd	—	—	bd
e	e	~	—
—	~	~	g
—	i	~	~

FIG. 2 — Antigenic factors in extracts.

these cases and none is indicated in the translation. After listing the factors indicated by positive results, a check is made among the negative results for conformation Thus, B ⟶ A ⟶ C and C ⟶ A ⟶ B suggest that B and C strains have no antigens in common, at least not those absent from A strain In case of contradictory results the negative result (absence of an antigenic factor) outweighs a positive result (presence of shared antigenic factors). The final list of factors is the minimum number accounting for the observed results. Following analysis of the factors for those experiments in which intact cells were used for immunization, the procedure is repeated for the results obtained when antigenic extracts were employed. Those factors which failed to survive the process of extraction or were partially inactivated are identified by an asterisk * in Fig 1

Following the analysis of antigenic factors shared by different strains it becomes necessary to introduce the factors « g h and i » in D C, and B strains respectively to account for the rejection of grafts from animals of these strains in recipients where no incompatibility was evident from the factors previously postulated.

Each of the latter symbols therefore, is probably referable to an antigen unique to the strain No corresponding symbol was introduced to account for the behavior of A strain homografts since rejection of these grafts on recipients of the other strains can be accounted for by other shared factors. Nevertheless it is probable that A strain animals do possess some unique antigens not found in any of the other strains. Following a general rule of parsimony in assigning symbols, none has been introduced where they were not necessary to account for the observed behavior The factors b and d assigned to the strains A and D could be collected and represented by a single symbol « bd » although the factors « a » and « f » in the strains A and C, also « c » and « e » in strains A and B are regarded as distinct by virtue of their behavior upon extraction

In two cases, the results are equivocal and in each of these it is the positive results that are incongruous The partially broken down grafts in the combination D→C→B may have resulted from undetected errors in technique. This explanation does not hold for the combination B→C→A, from which the presence of an antigen present in B strain but not all A strain animals, was indicated Although there was no other evidence of inhomogeneity in A strain this possibility was investigated and in fact, a segregating gene has been found in that strain which seems to offer the explanation Its presence did not influence the results in other combinations, and it did not show up in the reverse combination because of the large primary donor pool used. This mutation is under current study and the results will be reported elsewhere.

Discussion

The experiments have demonstrated the methodology for typing transplantation antigens. In principle, it is quite analogous to that practiced in the serologic typing of the red cell iso-antigens Both depend on the evocation of immunity by the exchange of antigens between individuals, followed by challenge of that immunity with antigens from other individuals whose relationship is to be established In serology the antibodies are drawn from the immune recipient and the challenge is made *in vitro*, in transplantation typing the challenge is made *in vivo* by means of the test homograft. Both procedures depend on exploring a variety of donor recipient combinations each resolving additional factors as different degrees of genetic relationship become exposed. To provide further fractionation serologic procedures make use of absorption to progressively reduce the antibody population the same sort of reduction can be brought about in transplantation typing by degradation of the primary antigens, as by extraction. Serologic typing has brought

an appreciation of the diversity of red cell iso-antigens in the present study it has been shown that the diversity of transplantation antigens is amenable to description by application of the same principles and a sample of that diversity has been exposed.

There is evidence that the H-2 locus may be composed of two or more units and that crossing-over between them leads to the segregation of antigenic factors. Definition of this region is difficult, however in the absence of genetic marker on each side of the locus. It is inviting to explain the polymorphism of complex loci of this kind (such as the Rh locus in man) in terms of a series of closely linked pseudo-alleles, each of which is reflected in the assortment of antigenic factors. On the other hand, there is little need to invoke more than the alternative genes at a single locus—multiple alleles—to account for the diversity and it is of importance to know which explanation holds.

As Billingham, Brent and Medawar [1] have pointed out each allele at the H-2 locus appears to produce both transplantation antigens and hemagglutinogens. Particular interest is drawn to any correspondence of the two kinds of antigens since serum antibodies against hemagglutinogens are able to compete in some manner with the transplantation antigens giving rise under appropriate conditions to a state of enhancement in which homograft rejection is delayed

If T and H antigenic factors are associated through identical units of inheritance, as the present facts indicate, there may be a unique opportunity to dissect the underlying genetic structure and the antigens themselves, now that the typing of T-antigens affords a « double-barreled » approach to the problem. Through correlation of H antigenic factors disclosed by serology and T-antigenic factors, revealed by transplantation typing the symbols relating the two sorts of antigens through common units of the genetic structure may be identified Those symbols which are artefacts of immunologic cross-reactivity a source of common confusion at present, should at the same time become evident.

Although the present study must be extended to include other strains before definitive data on the H-2 locus become available the results leave little doubt of the importance of transplantation antigens associated with those H-2 alleles thus far sampled Although there are only 6 experimental reactions where H-2 incompatibility was not anticipated on the basis of known H-antigenic factors, all of these results were negative However positive results were obtained for the one control reaction in which no H-2 incompatibility was expected. With this single exception all [illegible] 6 positive reactions fit within the boundaries imposed by I [illegible] It is noteworthy that no reactions were brought out [illegible] the presence of the H-factor « C » also that reactions corr[illegible]

associated with the factor « F » were attributable to immunological cross-reactions.

The constitutions of the strains B and D require comment It was assumed at the outset that the two strains were co-isogenic (IR) mice differing only at H 2 During the course of the experiments, the genetic disparity evidenced by the proportion of surviving parental grafts on the F-2 progeny of crosses between the strains was found to include no less than 4 genes in either direction. Even though the strains had been propagated by sib mating for many generations, the failure to maintain continuous selection and back-crossing to insure co-isogenicity resulted in divergence at several loci.

It is of interest, nevertheless that antigens associated with the common genetic background of the strains B and D which must include about a dozen shared genes were hardly perceptible. These common factors would have shown up as positive results in the four combinations B → A → D D → A → B B → C → D and D → C → B and, in fact a few positive results were noted. Because of the failure to observe consistent positive results when the combination B → C → D was reversed the results in that combination were attributed to cross-reactions. The findings do illustrate the point that the early rejection of grafts seems to depend on immunity against a few strong genes and in the case of these strains probably only those at the H-2 locus were involved. The tests are soon to be repeated with different co-isogenic sublines of C57BL.10/Sn and B 10/D-2 Sn generously provided by Dr George Snell. Breeding stocks of the sublines used here came originally from the Borges colony by way of the National Institutes of Health

Concepts of the specificity of transplantation immunity have, in the past, lacked precise meaning mainly because there has been so little information on the subject. It is not improbable that past failures to observe the expected overlapping of transplantation antigens may be attributed to the fact that perceptible levels of immunity are rarely reached against the largest number of these antigens, at least by the usual means of immunization. In order to gain some understanding of the immunologic specificity of the responses it is obviously necessary to eliminate those sources of apparent cross-reactivity that have their explanation through shared antigens. From the present results, it would seem that the antibodies causing homograft rejection behave like other orders of antibodies they are probably no more nor less specific. Several recent investigations [2, 3] dealing with the identification of transplantation antibodies, point up the urgency of physically separating individual antigens from the present crude extracts. Because these antibodies can be identified and defined through their reactivity with the antigens, it is evident that our understanding of the two must proceed hand in

hand. In this connection the applicability of typing procedures is worthy of comment.

It should be evident that several different antigens may result from the inheritance of a single gene; further that several antigenic factors may be identifiable with each kind of antigen. It is also possible that several antigenic determinant groups may be found in residence on a single kind of carrier molecule or that each individual antigen may be a distinct kind of molecule. For the same reason that serologic typing depends on reducing the antibody population by absorption, the preparation of transplantation typing reagents depends on reducing or fractionating the population of antigenic specificities. From the present results — the fact that the antigens do not survive equally during extraction suggests that they may have distinct physical and chemical properties. If this is true, a number of fractionation procedures can be applied to the problem; however, they depend on means for following the activity of each of the various antigenic components. It is obvious that so long as one kind of antigen survives in each fraction it will appear as though a separation procedure was ineffective.

Deliberate attempts to accomplish the same sort of reduction by progressively inactivating antigens, as through enzymatic digestion and other selective agents, also require typing of individual antigenic factors. Thus so long as a particular treatment does not affect all of the antigens it will appear as though it was ineffective when in fact certain of the antigens may have been susceptible to the treatment. In these and other manipulations with transplantation antigens it is apparent that typing will provide important and necessary information.

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SUMMARY

When mice of one inbred strain are immunized by primary exposure to transplantation antigens from another strain and subsequently challenged with homografts from a second donor strain, such grafts are frequently rejected prematurely, depending on the genetic relationships of the strains. The result can be taken as evidence that the primary and secondary donor strains share in common histocompatibility genes, hence the corresponding antigens that the recipient lacks.

All of the permutations (36) of primary donors, secondary donors and recipients possible with four strains of mice were tested for shared or related antigens. In this way it was possible to identify several antigenic factors and determine their distribution among the strains when spleen cell suspensions were

used for primary immunization. When antigenic cell free extracts were used for immunization fewer factors were in evidence. It was concluded that transplantation antigens are quite probably a diverse group of substances having distinct physical and chemical properties as well as antigenic specificities. The methodology of transplantation typing is discussed, and the findings are related to the diversity of hemagglutination factors controlled by the H-2 locus.

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DISCUSSION

GORER I would like to congratulate Dr Berrian on a very fine piece of work I do not want to start a battle with Dr Owen or with anybody else, but I do think that this question of cross-reactivity can be overrated. Now in most immunological reactions a cross reaction appears undistinguishable from a quantitative difference. And I do not quite see how an *in vivo* test is going to be any better than anything else because it seems that if you cross a road from north to south you should be able to cross it from south to north And I do not see quite why Dr Berrian's test should be better or worse than an *in vitro* one. Dr Berrian and I went through his results last night and, in general his results fit the known H-2 components very well the weak ones are weak in his test. Those components which I was rash enough to think might not exist, he was kind enough to show probably do not exist, which was very gratifying One thing does interest me very much in his table II where he is dealing with the H-2K antigens they seem to function very well with cells and be very unstable in the extracts except for the E components. That was extremely interesting to me, and it does perhaps offer some other way of finding more information on these different components.

I really do agree with Dr Berrian that *it is most important to* concentrate on the strong components it does not really matter much about these little weak ones, which as he says you can pile up until you get tired of it I think it is a very excellent piece of work and I hope he will continue it

BERRIAN The *in vitro* tests Dr Gorer is referring to describe the factorial distribution of H-antigenic factors, whereas the present tests deal with T-antigens. I have suggested that by comparing the distribution of both sorts of antigens we may be able to map any units of inheritance they share in common in the fine structure of the H-region Description of H antigens or T antigens alone is likely to give an unrealistic estimate of the complexity because of cross-reactive antibodies I would not expect the patterns of immunological cross reaction to coincide since the antigens are not likely to be identical and different channels of response appear to be involved

BRENT There is just one point I would like to raise and that concerns the sensitivity of this particular test for the presence of antigens. Dr Berrian used antigenic extracts which elicited a very strong immunity against the strain from which they were taken Now does this not mean that he might very easily miss even quite strong antigens which would be obscured by the strong immunity elicited by others? I wonder whether due to the flatness of the immunity response curve the test might not be made more sensitive if he uses an antigenic stimulus which results in a relatively feeble immunity against the strain used for immunization? My suggestion applies even more strongly to the weak tissue antigens. I am of course assuming that the effect of different antigens in one extract is additive

BERRIAN I fully agree with Dr Brent that a number of antigens may not have been resolved in the present test pattern however I doubt that weaker immunization is likely to bring out weak antigens that are obscured by the presence of stronger ones One has to eliminate the stronger factors to disclose weaker ones and this can be done by employing additional recipients, secondary donor combination or by inactivating the stronger ones. Having done this one would then like to ensure a strong immunity so that the test graft surviving in the presence of a violently rejected control graft will provide convincing data that the primary and secondary donor share no factors absent from the recipient.

There is little evidence that weak antigens have an accumulative effect and so far as I know the rates at which immunity develops against

the various members of a collection of antigens are independent of each other If this assumption is correct then the graft will be assaulted first by antibodies arising to the strongest factor and the effect of later responses will be of little consequence.

OWEN I would like to ask Dr Berrian whether in the combinations in which he did not observe pre-immunization there is any evidence of enhancement Did you follow the survival of these grafts beyond the time when they would have been expected to have been sloughed on the basis of a primary reaction?

BERRIAN No unfortunately this was not done for lack of cage space and other reasons. In the future it will be of importance to look for any extension of survival beyond the normal MST's.

OWEN It does seem to me it would be a matter of considerable interest if for example, the disappearance of transplantation effect from the extract is reflected in a diversion of an antigen in the direction of producing an enhancing type of antibody

BERRIAN Dr Owen's important suggestion is an extremely inviting avenue for further research

Ovarian transplantation in mice

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It is generally accepted that the outcome of the transplantation of a tissue from one animal to another can be predicted in terms of a series of genetic « laws of transplantation » which have been set out by Snell [11]. Of these, the most important generalization is that the host cells must contain at least all the histocompatibility genes which the donor tissue possesses. Whether they contain other histocompatibility genes as well is of no consequence when skin is grafted though it may be important if the graft contains cells which are able to react against the host. Apart from Bittner's [3] notable work with transplants of normal spleen, the early efforts to determine the number of such genes by immunogenetic analysis were almost entirely confined to the requirements of tumour transplants. Barnes & Krohn's [1] experiments represent the first full scale attempt to apply the genetic form of analysis to the normal tissue which is most commonly transplanted, i.e. to skin.

It might be thought that other normal tissues would have histocompatibility requirements similar to those of skin, and indeed the experimental conditions in which « immunological tolerance » can be obtained imply that skin, spleen cells and bone marrow cells each have identical complements of antigens and presumably of genes. On the other hand, it is not inherently impossible for other organs to have differing histocompatibility requirements just as some tumours seem to be less demanding than others. The endocrinological literature in particular contains numerous reports that grafts of endocrine glands succeed in circumstances which would certainly be expected to be unfavourable for the survival of a skin graft. Much of the evidence that endocrine tissues are less exacting in their requirements or that the host deals more leniently with them becomes less convincing when it is carefully exa-

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mined (see review, Krohn [7]). Nevertheless it purports to show that the fate of an endocrine graft, though dependent to some extent on genetic factors, is also considerably influenced by other factors such as the hormonal environment, the presence or absence of other endocrine glands, the « need » for the gland's hormone, the rate of growth, or the age of the graft, and more specifically, on evidence that ovarian grafts may survive in circumstances in which skin grafts fail.

The experiments to be reported here can be divided into two main parts. In the first, the behaviour of ovarian homografts exchanged between mice of various inbred strains is examined. In the second, the same method which Barnes & Krohn [1] used in their work on skin has been applied to the estimation of the number of histocompatibility genes governing ovarian transplantation.

The ovary was chosen to represent the members of the endocrine system because of the relative ease with which its function can be assessed. Since the ovary has not only an hormonal but also a gametogenic function, any complete assessment requires the transplant to be made orthotopically. In general, however, tests of endocrine function are sufficient for the purpose of establishing whether the graft survives or not. To do this it is enough to transplant the ovary to the subcutaneous tissues and to use continuance of cyclic cornification in vaginal smears as the index of survival. To this test can be added the information provided by the histological condition of the graft and of the uterus after removal at autopsy.

Materials and methods

Animals used.

All the mice used were bred in our own colony. The Strong A and CBA mice which provided the F_1 hybrids and donor material were of the same lineage as those used previously by Barnes & Krohn [1]. The female F_2 progeny, derived from mating F_1 littermates, were used when they were 40-80 days old.

The only non inbred strain used was the L.A.B. Grey strain of which the Laboratory Animals Bureau provided a breeding nucleus.

Surgical procedures.

The mice were anaesthetized with an intraperitoneal injection of Avertin (0.2 ml of a 1.25 % solution in normal saline per 10 g body weight). Both ovaries and small pieces of the upper part of the uterine horns were then removed through bilateral lumbar incisions. The ovaries to be grafted had recently been removed from the donor; each was

Ovarian transplantation in mice

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It is generally accepted that the outcome of the transplantation of a tissue from one animal to another can be predicted in terms of a series of genetic « laws of transplantation » which have been set out by Snell [13]. Of these, the most important generalization is that the host cells must contain at least all the histocompatibility genes which the donor tissue possesses. Whether they contain other histocompatibility genes as well is of no consequence when skin is grafted though it may be important if the graft contains cells which are able to react against the host. Apart from Bittner's [2] notable work with transplants of normal spleen, the early efforts to determine the number of such genes by immunogenetic analysis were almost entirely confined to the requirements of tumour transplants. Barnes & Krohn's [1] experiments represent the first full-scale attempt to apply the genetic form of analysis to the normal tissue which is most commonly transplanted, i.e. to skin.

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cut into two and stored in a sterile Petri dish on filter paper moistened with saline until required A bisected ovary was transplanted to each groin so that the pieces lay close to the femoral vessels at the ends of small tunnels under the inguinal pads of fat.

The technique of orthotopic transplantation will be described elsewhere (Krohn [8]).

Skin was transplanted in accordance with the technique previously described by Billingham & Medawar [3]

Hormones

Chorionic gonadotrophin dissolved in saline was given by subcutaneous injections of 10 units daily for two days.

Assessment of function of graft

Vaginal smears were taken daily in the usual way with a wire loop and stained with a mixture of methyl blue and eosin. Oestrogenic activity of the grafts was judged to continue as long as smears were found which contained no leucocytes but only cornified or nucleated epithelial cells or both types of cell together

Vaginal smears were taken for at least 28 days after the last period of vaginal cornification before deciding that the grafts were no longer alive. This interval was sufficient to allow the uterine horns of one group of F_1 mice which had destroyed their grafts to atrophy so completely that they weighed no more than the uteri of another group of spayed F_1 mice which had not received grafts.

An immediate difficulty was the establishment of a criterion by which to judge permanent survival of a graft A period of 100 days was used earlier when assessing the survival of skin grafts and this figure has again been used although it must be recognized that ovarian grafts, like skin grafts are occasionally destroyed after intervals of more than 100 days from the day of grafting The figure (in days) given in the tables for the survival time is the number of days elapsing between the day of grafting and the last day on which a fully cornified vaginal smear was found.

The survival of skin grafts was judged visually by the usual naked eye inspections at frequent intervals, and by histological preparations.

Procedure at autopsy

At autopsy the grafts were removed and fixed in Bouin's fluid for histological examination The sites of ovariectomy were inspected closely for evidence of any ovarian remnants and the size vascularity and condition of the uterine horns was noted.

PART I — THE FATE OF OVARIAN HOMOGRAFTS EXCHANGED BETWEEN STRAINS OF MICE.

RESULTS.

Small preliminary experiments, about 45 in number using reciprocal combinations of C_3H R III, A CBA CBA × A and L.A.B Grey mice need not be described in detail but in no instance was there any sign of an oestrous vaginal smear There was some histological evidence of survival in grafts removed 10 days after operation but none in any of the grafts which were removed after 20 days. Numerous control observations have established that isografts within all the various strains used, and also to the F_1 hybrids are uniformly successful

A larger experiment was then carried out in which reciprocal combinations of R III C_3H and L.A B Grey mice were used groups of five animals being killed at 6 12 18 24 and 30 days after grafting and smeared for 6 days before death (last 4 groups) The results are given below in Table I from which it is clear that grafts do not survive in such circumstances where the genetic differences between individual strains are wide. A very occasional positive vaginal smear more than 12 days after grafting may be observed when a non inbred L.A.B Grey mouse is the host but not when it is the donor Even in these instances the uterus at the time of autopsy was atrophic and the grafts like all the other grafts showed the histological signs of a typical homograft reaction

TABLE I — *The proportion of cornified vaginal smears in spayed mice receiving ovarian homografts*

Days after grafting on which smears were taken.	Proportion of vaginal smears found in oestrus					
	Donor strain					
	RIII		C3H		L.A.B Grey	
	Grey	C3H	Grey	RIII	C3H	RIII
7 12	6/30	0/30	0/30	0/30	5/30	5/30
13 18	3/30	0/18	2/30	0/30	0/30	0/30
19 24	1/22	0/18	1/24	0/30	0/30	0/13
25 30	1/24	0/18	1/24	0/30	0/30	0/30

Injections of chorionic gonadotrophin were given to further groups of mice 24 and 30 days after grafting, to see whether any evidence could be obtained for the survival of small groups of cells that might be sti

mulated by the hormone to produce oestrogen. None of the injected mice ever showed any signs that ovarian tissue remained alive

In a further group of animals, the grafts were removed 20 days after grafting and replaced by a second pair of ovaries which were allowed to remain for another 21 days. It was hoped to compare the number of positive smears derived from the hormonal activities of the two sets of grafts and to show *perhaps that the second set of grafts was less* active and broke down more rapidly However no oestrous smears were obtained after either the first or second ovarian grafts, and consequently there could be no evidence for an accelerated reaction to the second set.

TABLE II. — *The fate of orthotopic ovarian grafts*

Donor strain.	*Host strain.*	*Proportion of successful grafts.*
A, CBA, A+CBA and CBAxA	CBAxA	11/11
CBA	A	0/1
A	CBA	0/1
CBAxA	A	0/2
CBAxA	CBA	0/1
RIII, C H and C HxRIII	C HxRIII	8/8
RIII	C H	0/3
C H	RIII	0/3
C HxRIII	RIII	0/3
C HxRIII	C H	0/2
Grey	CBAxA	0/5
CBAxA	Grey	0 10

The fate of orthotopic ovarian grafts

The results of some experiments are summarized in Table II Success is based on the vaginal smear record and on histological examinations. Although all the mice carrying compatible grafts mated and became pseudopregnant, not all became pregnant. The technical reasons for this failure need not be discussed now but the complete absence of any sort of evidence of survival when the graft might be expected to fail on genetical grounds should be noted. Numerous other experi-

ments not reported here, have shown that isografts between members of several inbred strains always succeed (at least to the extent of producing hormones) but that grafts in non-inbred strains do not

The capacity of the ovary to act as an immunizing agent

One explanation for the exemption of the ovary whether it be partial or complete, from the usual homograft reaction, is that the graft does not produce an adequate antigenic stimulus or one which, for some anatomical reason of lymphatic drainage perhaps, fails to arrive in sufficient strength at the immunity producing centres An experiment was therefore carried out in which groups of C_3H and A strain mice received one or two sets of ovarian homografts from the other strain (with an interval of 27 days between the two grafts) and then received a skin homograft on the 47th day of the experiment. All these skin grafts were destroyed by the sixth postoperative day and histological preparations revealed typical « second set reactions » It is evident, therefore, that an ovarian graft can induce as complete a state of immunity as can the more usual skin graft. There is no reason to question its antigenic powers.

PART II — THE ESTIMATION OF THE NUMBER OF HISTOCOMPATIBILITY GENES CONTROLLING THE TRANSPLANTATION OF THE OVARY IN MICE.

Despite this clear evidence that ovarian grafts in mice succumb to the usual sort of homograft reaction there are other observations on rats (Parkes [19]) which seem to show that ovarian grafts exchanged between members of two closed but not inbred colonies of rats succeed more often than might be expected. Billingham & Parkes [4] have also compared the expectation of survival of skin and ovarian grafts exchanged between members of one of these two closed colonies. Of 31 rats given skin grafts 17 had completely broken down by the twentieth day On the other hand oestrous cycles continued for 61 days or more in 16 out of 19 spayed rats with ovarian grafts. When both skin and ovaries were transplanted together the ovaries survived longer than did the skin

One of the possible explanations for this finding is that there are fewer histocompatibility genes controlling the transplantability of the ovary than there are for the skin. An attempt has therefore, been made to estimate the number of such genes in A strain mice so that the figures for the ovary and for skin may be compared (*)

(*) The results of similar experiments using CBA mice are nearly complete and should be available at the Symposium.

TABLE III — *Distribution of survival times of A strain ovarian grafts and skin grafts* (Barnes & Krohn [1]).

Survival time (days)	No. of F mice		
	Ovarian grafts		Skin grafts.
0 10	77	= 61 %	
11 15	15		61 = 51 %
16 20	6		29
21 - 25	9		19
26 30	4		4
31 35	5		4
36 40	6		—
41 50	7		1
51 60	3		—
71 80	2		—
81 90	1		—
> 100	14		2
Total	151		120

RESULTS

The distribution of survival times of A strain ovarian grafts transplanted to 151 spayed F_2 mice is given in Table III It will be seen that cycles of vaginal cornification persisted for more than 100 days in 14 mice. This figure appeared to be unexpectedly high for on the basis of the work with skin no more than two or three survivors were to be expected. A comparison of the distribution of all the survival times also showed that the proportion of ovarian grafts surviving for more than 30 days was considerably higher It was, therefore decided to test each mouse which had accepted the A strain ovary with a graft of A strain skin The results of such tests showed that Strong A skin was always acceptable to those F_2 mice whose acceptance of an ovarian graft had already selected them out of the total population of F_2 mice as immuno-genetically compatible with the A strain

Of this group of fourteen F_2 mice thirteen have so far received skin grafts (usually about 130 days after the original ovarian grafts) Seven (PII 45 49 63 177 187 245 353) have shown complete compatibility

with both skin and ovarian grafts, which remained entirely healthy for more than 80 and 200 days after grafting respectively

Although the other seven ovarian grafts fulfilled the 100 days survival criterion two were not entirely healthy at autopsy and four broke down completely after surviving for more than the 100 days and one had almost broken down. These slight incompatibilities are reflected in the concordant behaviour of the skin grafts which they received For example, one mouse (PH 101) showed irregularities in the oestrous cycles and a tendency towards prolonged cornification on the one hand with either minute surface scabs or niggling lymphocytic reactions in the skin grafts. In one (PH 35) the biopsy of the ovarian graft taken on day 142 indicated that a reaction had recently begun Vaginal cornification ceased and the skin graft was destroyed in about 20 days. A second biopsy of the ovarian graft another 20 days later showed that it had deteriorated further Nevertheless it was not wholly destroyed and some groups of oocytes were still to be seen in it. On the other hand the findings in PH 133 show that very infrequent and irregular oestrous cycles and a very severe reaction in the ovarian graft may be associated with a skin graft which still bears a reasonably good crop of A strain hairs.

TABLE IV — *The survival times of A skin grafts transplanted to F_1 hybrids whose ovarian grafts had been destroyed*

No. of mouse	No of periods of vaginal cornification.	Last day in oestrus.	Day of skin grafting	Survival time of skin graft (days)
123	4	41	79	11
125	6	39	79	20 24
183	2	41	78	14
161	2	39	78	12
191	3	34	77	13
259	7	28	82	11
118	6	87	101	27 33

More vigorous reactions occurred in the other mice. One (PH 243) provided skin and ovarian grafts both of which broke down at about 125 135 days. The ovarian graft of PH 345 broke down at 125 days but the skin graft was still intact 120 days later Finally in PH 349 transplantation of skin of the 130th day was followed by the break down of both grafts about 40 days later From these results it can be

decided that an ovarian graft does not necessarily resist the niggling type of reaction better than does skin. The final destruction of either may precede the other by a short interval but usually their ends are contemporaneous or the extent of the damage to each about the same.

TABLE V — *The survival time of A strain ovarian (second set) and skin grafts transplanted to F_2 hybrids whose first ovarian grafts had been destroyed.*

No. of mouse	No. of periods of vaginal cornification.	Last day in oestrus.	Day of 2nd set graft.	No. of periods of vaginal cornification.	Survival of second set ovary	Survival of subsequent skin graft.
281	5	55	62	0	nil	—
293	2	23	81	0	nil	—
341	8	93	131	0	nil	8
247	5	30	62	2	44	16
249	7	78	62	20	118	85
295	9	61	150	21 +	137 +	—
206	9	50	150	22 +	137 +	—

The response of F_2 mice to skin grafts transplanted after ovarian grafts had broken down.

The intention here was to study the extent to which the reaction against the first ovarian graft might modify the behaviour of the host towards a skin graft. The response was studied in two groups of mice, one in which the earlier ovarian grafts had been rejected immediately and without giving rise to any oestrous cycles at all and one in which they had been accepted for a limited time only. In the first group of six mice all the skin grafts had clearly broken down several days earlier when they were first inspected 11 days after operation. The relationships between the survival times of the skin and ovarian grafts in the second group is set out above (Table IV). It is clear that although the skin grafts were all rejected there is no evidence that the reaction was notably accelerated.

It seems therefore that an F_2 mouse which immediately rejects an ovarian graft from one of the parent strains is completely immunized against a subsequent skin graft from the same strain. Only a partial *immunity develops* however if the F_2 mouse accepts the ovary for some time before finally destroying it.

Two mice in this group (PH 123, 125) received a second pair of ovaries after the skin graft had broken down. This time neither showed any signs of vaginal cornification afterwards, and histological examination of the grafts indicated that the tempo of the reaction against them had been accelerated

The response of F_2 mice to second set ovarian grafts transplanted after the first set had been destroyed

As in the preceding section the intention was to study the changes in behaviour following the rejection of the first grafts. Experiments were only carried out on mice which had shown some vaginal response to the original graft

The results are set out above (Table V). There is some correlation between the number of vaginal cycles caused by the first graft and the behaviour of a second graft in that the second graft tends to break down more rapidly if the survival of the first graft was not prolonged. On the other hand, there is some indication that a process possibly akin to « enhancement » may occasionally occur since three of the second grafts have survived for a very unexpectedly long time. It is also notable that, where the second ovarian graft broke down rapidly, a subsequent skin graft behaved as if it had been transplanted to an immune animal. On the other hand the skin graft lasted much longer in those mice which had taken a long time to throw off the two ovarian grafts.

Discussion

None of the work reported in Part I lends any support to the idea that interstrain ovarian grafts, whether orthotopic or not, in mice are less susceptible to the homograft reaction than are skin grafts, although the vagina of an occasional mouse may still remain cornified for a little time after the surface of a skin graft breaks down. The failure of homografts in mice has been the general finding of all those who have worked with this species (Strong [12] Nordholt [9] Krohn [8] Parkes [11]).

It appears that an ovarian graft, despite its small size is fully capable of eliciting a homograft reaction against itself, and has no special dispensation which permits it to ignore the reaction. Furthermore, the degree of immunity which it elicits is fully sufficient to cause a subsequent skin graft to be rejected just as rapidly and as completely as if the first immunizing graft had been provided by skin rather than ovary

It can be calculated from the proportion of ovarian grafts surviving for more than 100 days that the number of separately segregating histo-

compatibility genes governing the successful transplantation of A strain ovaries is about eight or nine. If one includes only the seven of these 14 mice whose grafts seem to have been completely compatible the figure for the number of genes rises to eleven These estimates clearly are lower than the figure of not less than 15 arrived at by Barnes & Krohn [1] for similar experiments using skin Not only the number of survivors but also the distribution of survival times is different At first sight therefore the observations would seem to support the suggestion that ovarian grafts are less demanding in their requirements and to be in line with the experiments on rats mentioned earlier But the crucial experiments, which appear to remove the apparent discrepancy in as much as it concerns any differences between the behaviour of ovarian and skin grafts are those which show that all the mice which completely accepted ovarian grafts also fully accepted a skin graft that was transplanted later In other words, the effect of the first operation was to select out of the total F_2 population just those mice which were immunologically compatible with A strain tissues. The success of the second test grafts with skin is then a confirmation of the compatibility of other donor tissues and indicates that ovary and skin do not differ in their requirements. This concordance of response holds not only for the fully acceptable grafts but also for those whose acceptance was something if only slightly less than complete

If any further explanation is required it can best be provided by pointing out that the criterion for breakdown of a skin graft is simply the destruction of its surface epithelium The dermal elements remain alive after this has happened. Perhaps if the dermis was an endocrine organ the secretion of a hormone by it could be demonstrated after the epithelium had broken down just as oestrogen can be demonstrated after the germinal epithelium of the ovary has gone Unfortunately there is no such test.

It has therefore been shown that the proportion of successful ovarian grafts is no different from that of skin grafts. But the difference between estimates of gene numbers from this and the previous study still requires explanation. The stocks of animals were closely related so that the group of F_2 hybrids should have been closely comparable in genetic make-up The proportion of coloured (black cinnamon and brown) mice in this second sample was however considerably greater than in the first, and if the reactivity of a mouse is in any way associated with its coat colour there might be overall differences between the two series The statistical errors of sampling are also, large when one is dealing with the small proportions to be found in this sort of experiment

Apart from the differences in distribution of survival time the two sets of observations show equally wide ranges of survival time. It has again been demonstrated that slight incompatibilities may take more than a hundred days to express themselves and that even when the body's reaction has at last developed sufficiently to destroy the first graft it can do no more to a second graft than throw it off at a tempo which though accelerated is still much slower than normal.

Barnes & Krohn[1] felt that a mouse which was semi tolerant for genetical reasons differed from one in which immunological tolerance had been induced by the injection of spleen cells in the way it rejected a skin graft. In Billingham Brent & Medawar's[2] mice the reaction seemed to be very protracted whereas in Barnes & Krohn's[1] mice it was almost as rapid once it had begun as the normal reaction It is not as easy to interpret the exact behaviour of ovarian grafts from the study of the vaginal cycle records as it is to study the skin grafts by repeated direct inspections. But on the whole the cycles peter out rather than end abruptly and to this extent they do not confirm Barnes & Krohn's earlier view of the behaviour of skin grafts.

ADDENDUM

The results of 105 experiments in which CBA ovaries were transplanted to ovariectomized F 2 mice were presented at the Colloquium. Nine mice accepted the graft for more than 100 days and were subsequently tested with skin As with the A-strain grafts a proportion (three or four) accepted both grafts permanently The others were rejected concordantly between 100 and 200 days after grafting These results closely resemble those already reported in the paper

A report was also made of attempts to induce tolerance to skin by the neonatal injection of testis cell suspensions. In two mice out of about 60 tolerance was induced and was maintained for as long as 100 days.

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DISCUSSION

WOODRUFF There are three comments I would like to make on this very interesting paper

The first about these oddities of endocrine survival I think everybody who studies endocrine grafts finds these things we have found precisely the same thing in rats with adrenal grafts and something very similar in guinea pigs with thyroid grafts. I would like to suggest that this might be a manifestation of the kind of thing I was trying to talk about yesterday You start with these four similar grafts by some accident of the amount of available nutriment one graft begins to regenerate a little ahead of the others and is in some way different I suspect from grafts that have been a little slow to do this Perhaps for this reason it may have acquired the sort of special privilege that I was trying to talk about earlier? Two other questions first what is the dose of testicular cells which Dr Krohn used in producing tolerance? and secondly why not thyroid grafts as a test? Dr Sparrow and I found that it is extremely easy with a collimated scintillation counter to study in detail the status of thyroid grafts in rats, and the findings correlate remarkably well with subsequent histology Incidentally apart from studying the functional activity of the graft the use of larger doses provides a convenient and simple means of doing the preliminary thyroidectomy

KROHN I agree that these oddities in the behaviour of the host to homografts are always turning up and as you rightly point out cells which provide the final graft that one sees histologically regenerate from a very small fraction of the original cells and may not be the same as the original family from which they derive

The dose of testicular cells was somewhere between 5 and 10 millions very mixed cells from mature testes containing spermatogonia spermatocytes, sperm heads sperm tails and other fragments.

I would agree with your proposal that one should try to use the thyroid as a test organ in rats But these sorts of experiments with F 2 animals are virtually restricted to mice where one has the difficulty of completely thyroidectomising them I do not know whether these counting techniques are satisfactory with mouse thyroid or remnants of mouse thyroid grafts I do recall one paper where the grafts were put into the subcutaneous tissues over the skull, and the regeneration of capacity to take up iodine was measured. A most extraordinary effect was that within about 24 hours, the graft although histologically in a deplorable condition was already capable of taking up iodine again

MITCHISON Four points

First of all the relationship between the cornification of the vagina and the survival of cells of your grafts. This seems to be a similar problem to the one which we are confronted with in transplanting immunologically active tissue which is making antibodies. Can you specify at all the relationship between the cell survival and the effects of the hormones which these cells produce?

Second If one takes a view that the survival of the original antigenic stimulus is important for maintenance of tolerance, it would be important that when you used gonad cells for inducing tolerance they should then survive until the test graft is made I wonder whether you have tried injecting trophic hormones during the early life of the mice to encourage the injected cells to survive and proliferate.

A third minor question which I have been interested in myself do you think there is any hope that the chromaffin test for adrenal medullary cells could be developed into so to speak a quick test for the survival of grafts, and one which did not require histology?

Fourth when you are looking for this very low proportion of long term survival grafts in F 2 s in different strains aren t you rather multiplying your difficulties? I am surprised that you have not taken two strains which we know to be very closely related for example a pair of Snell s co-isogenic strains where there is an expectation of getting a higher frequency of the more compatible mice than the F 2

KROHN It is just possible that a few ovarian cells can survive in insufficient quantities to produce enough hormone to affect the vagina We have controlled that possibility by always removing the grafts 28 days after the last period of vaginal cornification and serially sectioning them to make sure that no surviving ovarian tissue remained So it is possible that the true survival time of the graft is the survival time that we have used plus an unknown number of days which is less than 28

The testis cell suspensions were a mixture of cells, and contained spermatogonia which presumably would be able to go on dividing to form more spermatogonia. We have not at any time injected gonadotrophin to provide an additional stimulus. As to the chromaffin test for the adrenal medulla I don t know the answer The adrenal medulla in any case is a very difficult tissue to transplant. Indeed a lot of people have said that it is the one endocrine organ which cannot be transplanted although I am certain that it can be done.

When we began to do the skin work we had to use what mice were available and this determined our choice for the later work with ovaries.

OWEN I think it might perhaps be worth mentioning here as a matter of interest a parallel to Dr Krohn's demonstration of functional ovarian homo-transplants in tolerant mice that St. Amand and Smith at Oak Ridge last year succeeded in establishing functional ovarian homo-transplants in adult mice that had been rendered chimeras by the injection of homologous bone marrow into irradiated recipients

HYATT We clinicians have been faced with the problem of trying to make this work at the level of the patient, and I should like to pass on some information as a matter of comment and for advice and criticism we have followed largely the lead of Gaillard and the observation of Toolan has indicated that human embryonic skin at about the third month of gestation will survive transplantation for a considerable period of time.

In two instances we have been able to get such embryonic ovarian material By virtue of ovarian disease in the two patients the anatomical conditions for survival of the oocyte and ultimate pregnancy are destroyed. We felt therefore we were trying to salvage the hormonal effect

We noted that in tissue culture there was a tendency of the cells to migrate out to form a corona two to three cell layers thick These were transplanted on to a square of autogenous peritoneum about one inch in area It was then transferred to the peritoneum of the recipient a site believed by Gaillard to have important advantages in deciding whether the graft is successful or not.

In the instance of a 21 year old female we could find no evidence of persistent ovarian function. We must remember that when we did have ovarian oocyte survival in the autogenous ovarian grafts the formation of a follicle subsequently in the non-orthotopic site choked off graft function.

KROHN The idea of being able to transplant embryonic endocrine organs has been one that has attracted clinicians for many years indeed. Occasionally hopeful reports have cropped up, but generally speaking the reports have been unfavourable In those circumstances where the reports have suggested some effect particularly of ovarian grafts the criteria have often been mainly psychological and no one has looked for the graft at biopsy later on. This has been particularly true of adrenal cortical grafts when they have been used in the treatment of Addison's disease. The Addisonian condition has improved, but when the patient has died for some other reason absolutely no trace of the graft has been found at autopsy So any improvement could not really

be attributed to the graft. I doubt very much whether the peritoneum is necessary to form a bed for an ovarian graft certainly, the subcutaneous tissues anywhere, or the capsule of the kidney, or under the pretracheal muscles seem to give perfectly satisfactory ovarian grafts from the hormonal point of view

MURRAY I would like to comment on another type of ovarian homotransplantation which has been carried out by Drs. Sturgis, Sommers and H. Castellanos using Algire's millipore membrane. They have worked with the mouse, the rat, the monkey, and man their criteria for survival have been influenced on blood level of follicle stimulating hormones the pituitary vaginal cornification, changes in the weight of the uterus, and finally the changes in the functions on removal of the graft combined with the histological study In brief they had excellent results with the mouse, with the rat and the monkey It was quite encouraging and they have done four girls with ovarian dysgenesis at the age of about 15 They implanted these millipore membranes beneath the rectus muscle and also into the broad ligaments, not knowing which site would be more important. The children have not shown changes in vaginal cornification except transiently: it seems that some of the preformed hormones get through the filters. They have not shown any clinical changes, but on removal of some of the filters, they do find viable cells In the lower animals on removal of the filters, they have seen loss of the evidence of graft function, namely the vaginal function with return of vaginal cornification and the weight of the uterus becomes that of a castrate So this is another example of the difficulty of applying studies in the lower animal to the higher animal. Moreover I think it is a good example of where cellular viability does not necessarily mean cellular function

KROHN As I recall the appearance of the grafts inside the chamber they were distressingly unovarian-like they were rather collections of nondescript cells, and it would be very difficult I think for anybody to determine their origin.

VOISIN Concerning the testis cell suspensions I would like to ask Dr Krohn which technique he used in order to obtain his cells, and whether only spermatogenetic cells were present or whether possibly mesenchymal cells were mixed with them.

KROHN The technique we used was really to treat the testis as if it had been a spleen and to proceed in the usual way leaving behind on the sieve the outer stromal covering of the testis, but necessarily includ-

ing in the suspension not only the cells of the spermatogenic line but also the interstitial hormone-producing elements and the few other cells which are deposited between the tubules and which don't appear to be normal interstitial cells There would be a very small fraction—I have no possible way of measuring it—of non-spermatogenic cells there. One recent paper has suggested that some of these interstitial cells can take up Trypan blue and it has been suggested that they represent reticulo-endothelial elements in the testes. But these are single cells scattered about the testes and I do not really believe that they make up a sufficient number to influence the situation

The immunity inhibiting role of the spleen and the effect of dosage and route of antigen administration in a homograft reaction

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Introduction

The work to be presented in this paper was undertaken to study the effects of dosage and route of antigen administration in a particular homograft-host system. A survey of the previous literature had suggest ed that a state of « immunological tolerance » might be induced by large quantities of antigen administered intravenously and it was hoped that by using a suitable tumor homologous host system this hypothesis might be readily confirmed and its parameters explored Splenectomy was carried out in experiments utilizing high antigen dosage in the hope of inhibiting further the immune reaction

Materials.

The biologic system utilized has been extensively investigated by Barrett [1] and consisted of the inbred BALB/cAn mouse as homograft recipient the DBA 49 sarcoma as the homograft and inbred strain DBA/1An defibrinated whole blood as antigen Whole blood (including the red cell fraction) has been shown to be highly antigenic in this system and serves as an antigen conveniently quantitated and easy to administer intravenously The DBA 49 sarcoma a tumor which ori

( ) Most of the work presented in this paper was accomplished while the author was a member of the staff of the National Cancer Institute National Insti tutes of Health, Bethesda, Maryland.

ginated in the DBA/2An strain has the capacity of growing in various percentages of *non immunized strain BALB/cAn animals*, depending upon graft size and site of inoculation. With this system it is possible to study influences which could not be observed in a system less critically balanced at the point between graft success and failure.

At the time of use the tumor was in its fiftieth to sixtieth transplant generation—always in mice of the BALB/cAn strain. The tumor was always transplanted by the same uniform procedure to the subcutaneous tissue on the backs of recipient BALB/cAn mice. Freshly excised tumor tissue was cut into fragments of approximately 2 mm diameter for inoculation by 13 gauge trocar. The resulting tumors were measured at subsequent intervals and an approximate average tumor diameter to the nearest millimeter was estimated and recorded for each mouse. All measurements in each experiment were made by the same individual who was unaware of the experimental treatment and the results of previous measurements.

Strain DBA/2An blood was obtained from adult female mice (retired breeders) by exsanguination following decapitation under Nembutal anesthesia. It was defibrinated by gentle agitation with glass beads and pooled for use as antigen. Blood was not stored but was diluted with varying amounts of 0.86 per cent NaCl and used within three hours.

Throughout this work all inoculations, whether of blood or of tumor, were carefully alternated among the various groups of animals.

The BALB/cAn mice used as recipients were males, three to four months of age which were obtained from the Central Animal Production Facility at the National Institutes of Health. They were maintained in transparent plastic cages six to eight animals per cage and were given water and Purina Laboratory Chow *ad libitum*. All animals were individually numbered.

Experimental procedures and results

Lowest antigen dosage

Preliminary experiments (unpublished) were performed to select an approximate lower dosage limit of antigen efficacy. The dosage selected was 0.02 cc. of a one in twenty mixture of defibrinated strain DBA/2An blood in 0.86 per cent NaCl. The recipient strain BALB/cAn mice were randomized into five groups: one group was inoculated with blood intravenously by tail vein (I.V.), another by subcutaneous inoculation (S.C.), one group by intraperitoneal administration (I.P.) and another by carefully placing the inoculum into the intracutaneous tissues so as to raise

a bleb (I.C.). The fifth group was not inoculated and served as a non immunized control group All inoculations of blood were accomplished with a small syringe and 23-27 gauge needle.

Twelve days after inoculation of the strain DBA/2An blood all of the mice of all of the groups were inoculated (challenged) with the DBA 49 sarcoma The fragments of the tumor were placed subcutaneously by 13-gauge trocar and the inoculations were alternated among representatives of each of the five groups of animals.

The resulting tumors were observed for a period of thirty days from the time of inoculation They were measured at various intervals and an average diameter for each tumor was noted These observations as recorded 22 days after tumor inoculation, are shown in Table I In addition the change with time of the average of the tumor-diameters (including 0) is presented in Figure 1 (The I.P and I.C. groups are omitted on the graph for greater clarity). The difference in tumor growth between the non-immune, control tumor group and the group inoculated with antigen subcutaneously cannot be considered statistically significant. However both of these groups were significantly different from the intravenously immunized group which showed the greatest response ($P<0.001$) Differences between the intravenous, intraperitoneal and intracutaneous groups were not significant.

It is concluded that at a level of antigen dosage so low that the subcutaneous route showed little or no effect, the intravenous route was markedly efficacious

Intermediate Antigen Dosage

This portion of the experiment was performed in an identical manner to that previously described for the lowest antigen dosage, except that the dosage of antigen was increased to 0.02 cc of a one-in ten dilution of defibrinated whole blood in 0.86 per cent NaCl.

The results obtained with this dosage of antigen are presented in Table II and in Figure 2 (I.C. and I.P routes are omitted in the graph for greater clarity). It can be seen that tumor immunity was increased in all groups as compared with the previous experiment, and that although the subcutaneous route remained the least efficacious there was no significant difference among the various groups. At this level of antigen dosage effective immunity was produced by all four routes of administration.

Highest Antigen Dosage

This portion of the experiment was essentially similar to those previously described The antigen dosage was increased to 0.50 cc. of a

TABLE I — *Grossly perceptible tumors (2 days after tumor inoculation) in mice immunized with the lowest antigen dosage*
(0.02 cc. of 1 in 20 dilution)

Route of antigen administration.	*Number of mice*	*Number with tumor*	*Per cent with tumor*	*Average tumor size (mm) including 0*
I. V	32	15	47	2.[illegible]
I. P	28	16	57	3.40
I. C	32	15	47	2.70
S. C.	32	26	81	5.37
Control	32	30	94	5.50

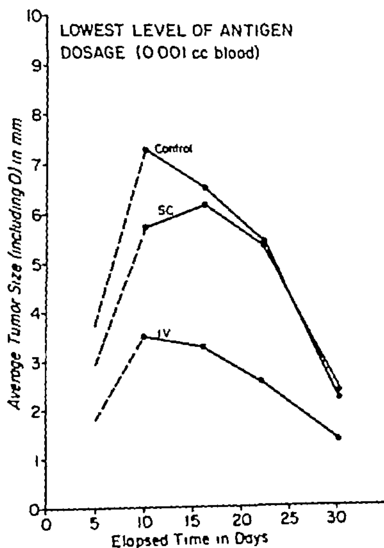


FIG. 1

TABLE II — *Grossly perceptible tumors (21 days after tumor inoculation) in mice immunized with intermediate-strength antigen* (0.02 cc. of a 1 to 10 dilution)

Route of antigen administration	Number of mice.	Number with tumor	Per cent with tumor	Average tumor size (mm) (including O
I. V	23	6	35	1.52
I. P	25	7	28	0.96
I. C	26	6	23	0.96
S. C	24	11	41	2.68
Control	27	23	85	6.14

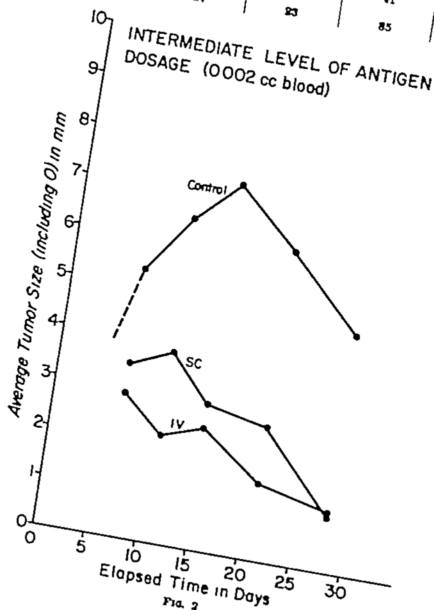


FIG. 2

one-to-one dilution of defibrinated whole blood in 0.86 per cent NaCl Only the subcutaneous and intravenous routes of administration were investigated but an additional variable, splenectomy was introduced Approximately one half of the animals of each group (including controls) were splenectomized under Nembutal anesthesia four to five days prior to immunization and sixteen to seventeen days prior to tumor inoculation. The remainder were subjected to sham operations. One-half of the non immunized control mice were inoculated intravenously with 0.50 cc. of saline while the other half were inoculated with the same dose of saline subcutaneously

TABLE III — *Grossly perceptible tumors (24 days after tumor inoculation) in mice immunized with the highest strength of antigen*
(0.5 cc. of 1 in 2 dilution)

Route of antigen administration and presence or absence of spleen.	*Number of tumors / Number of mice ( )*		*Per cent with tumor*	*Average tumor size (mm) including O*
	Exp a	*Exp b*		
I. V (sham operated)	17/17	8/9	78	9.5
I. V (splenectomy)	8/17	3/10	41	1.8
S. C. (sham operated)	0/16	—	0	0
S. C. (splenectomy)	0/16	—	0	0
Control (sham operated)	16/16	—	100	4.9
Control (splenectomy)	12/14	—	86	6.2

( ) In the intravenously inoculated groups the data from two separate experiments are presented These data have been combined in columns 3 and 4 and in Figure 3

The data are presented in Table III and Figure 3. The intravenous portion of the experiment was performed twice (although on a very small scale) with similar results as shown in Table III and the results of the two experiments have been combined in the graph The subcutaneous inoculation of antigen at this dosage produced a very solid immunity Splenectomy was apparently without effect in both the subcutaneously immunized and non immunized control groups and these results have been combined for greater clarity in Figure 3 However in the intravenously inoculated animals *splenectomy led to an immune status* in intact animals the intravenous inoculation produced enhanced growth of the challenge tumors The immunity produced by splenectomy was exactly the reverse of what had been anticipated!

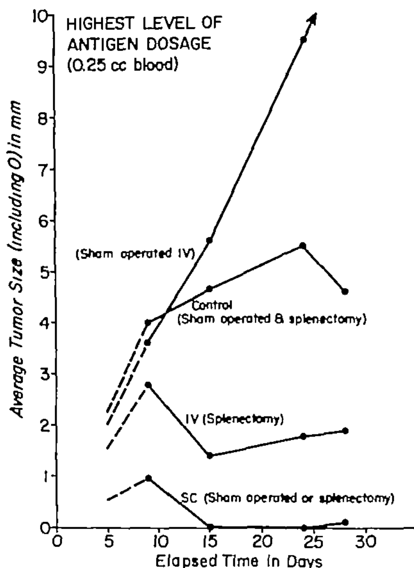


FIG 3

Because of the vigorous growth of the challenge tumors in the intravenously inoculated group a further experimental procedure was instituted Forty five days after their inoculation the tumors were excised from all remaining mice of the sham-operated control group and the sham-operated intravenously inoculated group Eight days later these animals, along with a new group of previously untreated controls, were inoculated with a new challenge-implantation of tumor The results of this second challenge as recorded 21 days later are shown in Table IV The animals were found to be immune to further tumor growth apparently immunized by the previous growth of the first challenge tumor inoculum

one to-one dilution of defibrinated whole blood in 0.86 per cent NaCl Only the subcutaneous and intravenous routes of administration were investigated but an additional variable splenectomy was introduced Approximately one-half of the animals of each group (including con trols) were splenectomized under Nembutal anesthesia four to five days prior to immunization and sixteen to seventeen days prior to tumor inoculation The remainder were subjected to sham operations. One half of the non immunized control mice were inoculated intravenously with 0 50 cc of saline, while the other half were inoculated with the same dose of saline subcutaneously

TABLE III — *Grossly perceptible tumors (24 days after tumor inoculation) in mice immunized with the highest strength of antigen* (0.5 cc. of 1 in 2 dilution).

Route of antigen administration and presence or absence of spleen.	*Number of tumors / Number of mice ( )*		*Per cent with tumor*	*Average tumor size (mm) including 0*
	Exp. a	*Exp. b*		
I. V (sham operated)	17/17	8/9	78	9 6
I. V (splenectomy)	8 17	3/10	41	1 8
S C. (sham operated)	0 16	—	0	0
S. C. (splenectomy)	0/16	—	0	0
Control (sham operated)	16, 16	—	100	4 9
Control (splenectomy)	12/14	—	86	4 2

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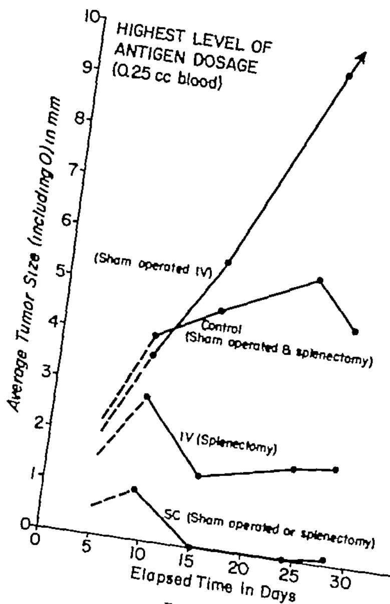


FIG. 3

Because of the vigorous growth of the challenge tumors in the intra venously inoculated group a further experimental procedure was insti tuted Forty five days after their inoculation the tumors were excised from all remaining mice of the sham-operated control group and the sham-operated, intravenously inoculated group Eight days later these animals, along with a new group of previously untreated controls were inoculated with a new challenge implantation of tumor The results of this second challenge, as recorded 21 days later are shown in Table IV The animals were found to be immune to further tumor growth apparently immunized by the previous growth of the first challenge tumor inoculum.

TABLE IV — *Grossly perceptible tumors (21 days after tumor inoculation) resulting from the second challenge of mice previously inoculated intravenously with the highest antigen dosage*

Experimental groups	*Number of mice*	*Number with tumor*	*Per cent with tumor*	*Average tumor size (mm) including O*
2d tumor inoculation (prior tumor excision and antigen intravenously)	17	2	12	0.33
2d tumor inoculation (prior tumor excision, no blood antigen)	13	3	23	2.5
1st tumor inoculation (no prior treatment)	14	10	71	4.8

DISCUSSION

The data presented in this paper are of interest primarily because of the paradoxical effect of splenectomy upon the results of the intravenous inoculation of large dosages of antigen. The other findings concerning the relative ineffectiveness of subcutaneous antigen at low dosage levels and the appearance of relative « tolerance » as a result of high intravenous doses was anticipated. Medawar [illegible], Billingham[4] and Snell[13] have presented somewhat similar data in the homograft context and much the same results have been obtained with a variety of heterologous antigens (Rowley[17]). While there has been much work indicating the role of the spleen in immunity production (at moderate levels of antigen [illegible]) the present data are to my knowledge the first to incriminate directly the spleen as an immunity inhibitor. Although the data are of preliminary nature they are adequate to establish statistical significance and there is no reason to believe that more data will seriously alter the results.

There would seem to be two possible mechanisms by which the spleen may produce an immunity inhibiting effect in response to large *doses of intravenous antigen* firstly the spleen may serve as a trap for the foreign material preventing its distribution in optimal fashion to *the various other sites of immunity production* secondly the spleen may actively produce an anti immunity substance perhaps a paradoxical antibody. *Further experimentation* should distinguish between these two hypotheses but in the absence of data to the contrary the second hypothesis appears the more probably correct. A simple trapping phenome

tion would not be compatible with the effective immunity achieved by smaller doses of I V antigen

An active antibody with paradoxical action on a homograft—especially a tumor homograft—has been demonstrated by Kaliss[9] and many of its properties have been investigated It seems probable that the enhancement of growth of the DBA 49 sarcoma is a similar phenomenon although the H-2 histocompatibility locus is not involved[11] Until this time, there has not been evidence that this particular tumor-host system would show an « XYZ » or « enhancement effect »[3 4] The specificity of the splenic action and the possibility of passive transfer of the effect by serum will have to be determined before this paradoxical antibody hypothesis can be established Perhaps the role of the spleen in other well known tumor « enhancement » systems should be investigated.

If one assumes that an « enhancement » antibody is responsible for the paradoxical splenic activity the question arises as to whether this activity is confined to the spleen or whether the spleen shares this peculiar property with other lymphoid or reticulo-endothelial organs Perhaps any antibody producing tissue would respond in this manner if exposed to a sufficient concentration of antigen but the intravenous approach to the spleen may be the only practical method of achieving this condition. One may speculate that perhaps a primary function of the spleen is as a second line of defence against the production of destructive auto-antibody Presumably the spleen exposed throughout life to large dosages of intravenously administered auto-antigen would automatically provide paradoxical type antibodies if there were any break down in the fetally acquired mechanisms[4] which normally prevent auto-immunization. One could speculate a step further and postulate that the paradoxical effect exhibited by the spleen may be a first line of defence and a principal part of the mechanism first activated in utero which prevents auto-antibody formation. This question could be posed experimentally by investigating what the effect of splenectomy might be on the process of induction of « acquired tolerance » in the new born mouse or rat[8] Although tumor « enhancement » and « acquired tolerance » appear to be distinctly different phenomena it is not yet certain into which category the spleen effect will fall.

Other related phenomena perhaps involve some splenic action. The « immunologic paralysis » of Felton[6] is more readily produced by the massive administration of antigen intravenously than by other routes. Is this because of the spleen?

There has been a clinical observation in man which may in some manner be related to the role of the spleen in suppressing some types of

immune response the observation by Dameshek[7] that lupus erythematosus may occasionally be enhanced or elicited following splenectomy

It would be possible to carry this kind of speculation on to greater lengths but no useful purpose would be served I have already gone beyond the limits of that type of mental rumination necessary to guide future experimentation Only more data can determine the actual role of the spleen in the prevention of auto-immune states and the import or lack of import of the data already discovered Perhaps it would not be amiss to point out however that a spleen which produced ordinary immunity to small or moderate amounts of intravenous antigen but which inhibited immune mechanisms in response to large doses would be admirable suited to the double task of protecting the animal from foreign invasion while at the same time preventing the civil war of auto-immune reactions

SUMMARY

The roles of antigen dosage route of administration and of splenectomy in a homograft reaction have been investigated in a system composed of the strain BALB/cAn mouse as homograft recipient, the DBA 49 sarcoma as the homograft and strain DBA/2An defibrinated whole blood as the antigen Results indicated that at a minimal antigen dosage the subcutaneous route of antigen administration was relatively ineffective while the intravenous and to a lesser degree the intraperitoneal and intracutaneous routes were efficacious At intermediate dosage levels, all four routes of antigen administration were effective At the highest levels of antigen dosage employed it was found that the subcutaneous route of administration produced a very effective immunity while the intravenous route produced an enhanced growth of challenge tumors Splenectomy at these high antigen-dosage levels was without effect when the antigen was administered by the subcutaneous route However in animals exposed to large doses of antigen by the intravenous route splenectomy prevented a state of enhancement and produced a relatively immune condition It is therefore speculated that the spleen may play an important role in the prevention of auto-immunization as well as in enhancement phenomena by producing a paradoxical or immunity-inhibiting antibody

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DISCUSSION

MEDAWAR The inferiority of the subcutaneous route in your system may be due to the fact that the red cell is antigenic, as Dr Barrett has shown. If the red cell is antigenic, one cannot infer from your results that the subcutaneous route would be equally inferior in systems in which the antigens are contained on highly mobile mesenchymal cells of one kind or another

PREHN This statement I agree with entirely

GORER I would like to mention a point of terminology which is especially pertinent to tumours but I think it does apply to normal tissues as well, and that is the use of the word « enhancement » There are several things which can enhance tumour growth. For example, some tumours can be enhanced by oestrogens. I think it is important to specify what type of enhancement you are referring to. Now in this case, it is not clear In the Kaliss situation it is because it can be done by means of antibody In the case of Professor Albert and of Dr Prehn, it is not clear It may be that they are dealing with Felton's type of paralysis which is the very reverse mechanism of the Kaliss effect. I have talked to Dr Prehn about the use of antibody and there is just one thing I would like to point out in case anyone wants to try it For some tumours the dose of antibody is extraordinary critical. You will only get enhancement over a certain dose range. If you put in too much you may get immunity

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It would be possible to carry this kind of speculation on to greater lengths, but no useful purpose would be served I have already gone beyond the limits of that type of mental rumination necessary to guide future experimentation. Only more data can determine the actual role of the spleen in the prevention of auto-immune states and the import or lack of import of the data already discovered. Perhaps it would not be amiss to point out however that a spleen which produced ordinary immunity to small or moderate amounts of intravenous antigen but which inhibited immune mechanisms in response to large doses, would be admirable suited to the double task of protecting the animal from foreign invasion while at the same time preventing the civil war of auto-immune reactions

SUMMARY

The roles of antigen dosage, route of administration and of splenectomy in a homograft reaction have been investigated in a system composed of the strain BALB/cAn mouse as homograft recipient the DBA 49 sarcoma as the homograft and strain DBA/2An defibrinated whole blood as the antigen Results indicated that at a minimal antigen dosage, the subcutaneous route of antigen administration was relatively ineffective while the intravenous and to a lesser degree the intraperitoneal and intracutaneous routes were efficacious. At intermediate dosage levels all four routes of antigen administration were effective At the highest levels of antigen dosage employed it was found that the subcutaneous route of administration produced a very effective immunity while the intravenous route produced an enhanced growth of challenge tumors Splenectomy at these high antigen-dosage levels was without effect when the antigen was administered by the subcutaneous route However in animal exposed to large doses of antigen by the intravenous route splenectomy *prevented* a state of enhancement and produced a relatively immune condition It is therefore speculated that the spleen may play an important role in the prevention of auto-immunization as well as in « enhancement phenomena by producing a paradoxical or immunity inhibiting antibody

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unfortunately enhancement certainly does not occur in these circumstances.

PREHN I think that, judging from the experiments I reported it is essential that the foreign antigen be circulated in a high concentration probably in the form of intact cells. The transplantation of a whole organ even of the size of the liver probably does not produce this condition and is more analogous to a subcutaneous transplantation. So that it would probably be necessary to transplant something such as bone marrow which would provide a high dosage of inoculating foreign cells.

PREHN One point about the dosage effect in searching for enhancement antibody we do have experiments under way to see if the effect we have described can be transferred passively with serum. Our experiments to date have been negative. However they have not yet been done adequately I am not at all convinced by what I have been able to read that there is necessarily a really distinct difference between the Felton type of paralysis and the ordinary Kaliss type of enhancement I am inclined to think that this may be more apparent than real. I would like an explanation from you as to what really is the essential difference between the Kaliss enhancement and the Felton type of paralysis.

GORER Well the main difference is supposed to be in the Felton type of paralysis antibody is not formed I do not think anybody has tried to transfer it passively with serum since there is apparently nothing to transfer And on the contrary you can protect a paralysed mouse passively with antibody against pneumococci provided you give enough antibody But they demand very large doses of antibody Now with a tumour like sarcoma I it does not matter how much antibody you give you will not protect. You may not make much difference to the enhancement, but that is the only phenomenon you will observe. And that I think is a rather big difference. It is a question of transference Kaliss has shown in experiments with cortisone that you will not get enhancement of sarcoma I if you abolish antibody production by means of cortisone. And that of course is very different from the Felton story

SIMONSEN Did you ever try to reinject cellular suspensions intravenously from your removed spleens to see if that would influence the enhancement you had from splenectomy alone?

PREHN As I stated, these are rather incomplete experiments and we certainly have this type of thing in progress

MERRILL Dr Prehn your hypothesis is an intriguing one for those of us who are interested in whole organ transplantation because if enhancement depends upon a large amount of tissue continually shedding antigen into systemic circulation transplantation of a kidney should of course be affected by this In other words the antigen is here in large amounts the cells do not die and yet splenectomy seems to make no difference Furthermore Dr Francis Moore on two occasions, I believe has successfully (at least in an anatomical sense) transplanted a liver about as large a « whole organ antigenic stimulus as we can get and

unfortunately enhancement certainly does not occur in these circumstances.

PREHN: I think that, judging from the experiments I reported, it is essential that the foreign antigen be circulated in a high concentration, probably in the form of intact cells. The transplantation of a whole organ, even of the size of the liver, probably does not produce this condition and is more analogous to a subcutaneous transplantation. So that it would probably be necessary to transplant something such as bone marrow which would provide a high dosage of inoculating foreign cells.

udies on the runting syndrome in newborn mice (*)

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Studies on the runting syndrome in newborn mice.

It has been reported by Billingham and Brent [1] by Simonsen [2] and Woodruff and Sparrow [3] that newborn mice rats or chicks treated homologous spleen cells frequently become sick and die by four s of age. These mice are smaller than normal mice of the same and show anemia [4] splenomegaly and focal coagulative necrotic lesions. This so-called « runting syndrome » or « homologous se » has been under investigation in our laboratory for the past This presentation will be in two sections (1) the discussion of con is necessary for the production of the runting phenomenon (2) the ssion of certain immunologic methods for protection of newborn against runting by homologous cells.

Experimental.

I — Conditions for production of the homologous disease

Types of tissues which will cause the runting syndrome Newborn d mice (of various lines) received intraperitoneal injections of saline nsions of various homologous and isologous tissues It was found homologous spleen cell suspensions and homologous lymph node uspensions caused runting while homologous liver cell suspensions logous kidney cell suspensions and isologous spleen cell suspen did not cause runting

(This work was supported by grants from th United States Publi Health B-1127 and H 2022 and from the Research and Development Divi ion Office Surgeon General, Department of the Army under Contract N DA-49-007)

Factors influencing incidence of runting — Three factors were found to influence the incidence of runting in inbred newborn mice receiving homologous spleen cells intraperitoneally

As seen in Table I, the incidence of runting depends upon the particular *strain combination* employed

TABLE I — *Incidence of runting with various strain combinations of mice*

Donor Strain.	*Recipient Strain.*	*Incidence of Runting ( )*
C57Bl/6	A/Jax	78/79 (99 %)
C57Bl/6	CAF	33/33 (100 %)
C57Bl/6	DBA2	78/85 (95 %)
C57Bl/6	Balb/C	38/41 (93 %)
C57Bl/6	Swiss Webster	33/40 (82 %)
C57Bl/6	CBA	16/16 (100 %)
C57Br	DBA2	14/14 (100 %)
CBA	A/Jax	9/20 (45 %)
Balb/C	DBA2	4 16 (33 %)
A/Jax	CBA	17/22 (77 %)

( ) Numerator indicates number of mice with runting syndrome
Denominator indicates number of animals in each experiment.

Although the majority of mice with the runting syndrome showed failure to grow at the normal rate and had liver necrosis when autopsied the criterion of runting used in all of these experiments was death of the animal between five days and thirty days of age Mice dying during the first five days after birth were not included in calculations of runting incidence as these early deaths could usually be attributed to trauma consequent upon the injection of the relatively large volume of fluid

As indicated in Table II the incidence of runting is related to the *dose* of homologous spleen cells given The greater the dose of spleen cells, the greater the incidence of runting

Finally we observed that the incidence of runting decreases as the *time interval* between birth and the administration of the homologo

TABLE II — *Influence of dosage of spleen cells in incidence of runting*

Donor Strain.	Recipient Strain.	Dosage of Spleen Cell Suspension ( )	
		0 05 ml.	0 01 ml.
C57Bl/6	DBA2	78/85 (95 %)	15/26 (58 %)
C57Bl/6 ..	A/Jax	78/79 (99 %)	2/6 (33 %)
C57Bl/6	Swiss Webster	33/40 (82 %)	7/50 (14 %)

( ) Spleen cell suspension prepared with three adult mouse spleens per ml.

spleen cells increases. *Our data, which confirm those of Simonsen*[4] are shown in Table III

Need for viable cells to produce runting — In order to determine whether viable cells were required to produce runting spleen cell suspensions which had been repeatedly frozen and thawed were injected into homologous newborn mice.

Spleen cell suspensions were prepared in the proportion of three C57Bl/6 spleens per ml of saline Half of each preparation was then rapidly frozen and thawed three times. Each litter of newborn mice was divided randomly into two groups one received the frozen thawed spleen cell suspension and the other received the untreated spleen cell suspension DBA2, CBA and A/Jax recipients were used The results as shown in Table IV indicate that the frozen thawed spleen cell suspensions did not produce runting Although two mice which had received frozen thawed cells died twenty and twenty-one days respectively after birth they were of normal size did not show the usual gross liver lesions seen on autopsy of runted mice and their death was not attributable to the experimental procedure.

It was also found that an homologous spleen cell suspension homogenized in a Potter Homogenizer would not produce runting when injected into newborn mice.

The cell free supernatant prepared by centrifuging a saline suspension of ground homologous spleen failed to produce runting when administered to newborn mice

Liver lesions in runted mice

In a large percentage of our runted mice we observed necrotic lesions in the liver These lesions were generally of a subcapsular distribution although some centrally located lesions were observed Histologically the lesions appeared as focal areas of coagulative necrosis with no or minimal cellular inflammatory reaction associated with the lesions

TABLE III.

Running incidence with spleen cell injection at various times after birth

Donor Strain.	Recipient Strain.	Age of Mice when Injected					
		1 ( )	2	3	4	5	6
C57Bl/6	Balb/C	38/41 (93 %)	19/24 (79 %)	5/10 (50 %)			
C57Bl/6	A/Jax	78/79 (99 %)	12/14 (86 %)		7/14 (50 %)		
C57Bl/6	CAF	32/33 (100 %)	15/24 (62 %)	13/14 (93 %)		6/16 (38 %)	0/11
C57Bl/6	DBA2	78/85 (96 %)	23/31 (74 %)	4/11 (36 %)	5/20 (25 %)	5 19 (26 %)	
C57Bl/6	Swiss Webster	33/40 (82 %)	6/16 (37 %)	8/75 (11 %)	12/68 (18 %)		

( ) 1 = day of birth.

II — *Protection of newborn mice against runting by immunologic methods*

According to Billingham and Brent [1] and Simonsen [4] runting is the result of foreign cells reacting immunologically against the host. The host receives the foreign cells on the day of birth at a time when it is immunologically immature, and becomes « tolerant » of these foreign cells [2]. We have attempted by three methods, to restore the immunological activity of the host against these foreign cells and thus protect against runting.

a) We first attempted to protect against runting by simultaneously treating the newborn mice with isologous adult spleen cells.

TABLE IV — *Effect of freezing and thawing of spleen cells on incidence of runting*

Treatment	*Incidence of Runting*
Frozen and Thawed Spleen Cells	2/20 (10 %)
Intact Spleen Cells	13/15 (87 %)

( ) These two animals died but were of normal size and had no liver lesion.

All the mice received 0.05 ml. of a suspension of three $C_{57}Bl/6$ spleens in one ml. of saline intraperitoneally within twenty four hours after birth. Each litter was then divided randomly with one half receiving no further treatment and the other half receiving 0.05 ml. of a suspension of four isologous adult spleens per milliliter intraperitoneally within thirty minutes following the injection of the homologous spleen cells. A few litters were divided into three groups: the first group received only homologous spleen cells, the second group received homologous spleen cells followed by isologous spleen cells and the third group received homologous spleen cells followed by isologous liver cells. Also several litters of mice were divided into two groups: one receiving homologous spleen cells only and the other receiving homologous spleen cells followed by isologous liver cells.

Balb C, CBA, A Jax and DBA2 mice were used as recipients. $C_{57}Bl/6$ mice were used as donors in all cases.

Our experimental results which are summarized in Table V indicate that isologous spleen cells given to newborn mice within thirty minutes following homologous spleen cells confer significant protection

against the runting syndrome None of the twenty-three mice which received only homologous spleen cells survived thirty days, while thirteen out of twenty-one or 62 % of the mice treated with isologous spleen cells survived and were well after thirty days. Those mice which died despite treatment with isologous spleen cells tended to live longer than did their untreated littermates. Our data (see Table V) indicate that isologous liver cells, given within one half hour following homologous spleen cells, confer no protection against runting All of our twenty mice treated with isologous liver cells died before twenty days of age.

TABLE V — *Protection by adult isologous spleen cells against runting syndrome*

Group.	Number of Mice	Number of Mice dying — Day of Death					Surviving 30 Days.
		6-10	11 15	16-20	21 25	26-30	
Newborn mice given homologous spleen cells	32	7	17	6	0	2	0
Newborn mice given both homologous and isologous spleen cells	21	0	0	4	4	0	13
Newborn mice given homologous spleen cells and isologous liver cells	20	5	12	3	0	0	0

TABLE VI — *Failure of isologous adult spleen cells to protect when given one day after homologous cells*

Group	Number of mice	Number of Mice dying			Surviving on day 30.
		Day 6-10	11 15	16-20	
Newborn mice given homologous spleen cells	15	5	10	0	0
Newborn mice given homologous spleen cells and isologous spleen cells one day after birth	19	3	11	3	2

In other litters, the above procedures were repeated with the exception that the injection of isologous spleen cells was delayed until one

day after birth and in these experiments we observed no significant protection against runting. As shown in Table VI all of our fifteen animals which received only homologous spleen cells died by twenty days of age as compared with seventeen deaths out of nineteen mice treated with isologous spleen cells one day after birth.

b) It was next attempted to protect animals against runting by treating the babies with serum prepared from mice immunized against the donor strain. It was felt that such serum might be active in destroying or permitting the newborn mouse to destroy the foreign spleen cells injected.

In this experiment C57Bl/6 mice were used as spleen cell donors *and DBA2 newborn mice as spleen cell recipients. Serum was prepared* by immunizing DBA2 mice against C57Bl/6 spleen cells. Each DBA2 adult mouse was inoculated with a suspension of C57Bl/6 spleen cells in complete Freund's adjuvant into the toe pads. Each animal received two inoculations spaced one week apart and was bled from the retro-orbital plexus one week after the second inoculation. This serum will be referred to as *DBA2 anti-C57Bl/6 serum*. Serum was similarly prepared from normal adult DBA2 mice. Newborn DBA2 mice were treated with C57Bl/6 spleen cell suspension within twenty-four hours after birth. Each litter was then divided: one half was treated with normal DBA2 serum intraperitoneally, the other half was treated with DBA2 anti-C57Bl/6 serum. The schedule for treatment of mice is indicated below.

Age in days	*Treatment*
1	received C57Bl/6 spleen cell suspension
2	no treatment
3	0.0[illegible] ml serum
4	0.05 ml serum
5	0.0[illegible] ml serum
6	0.10 ml serum
7	0.10 ml serum
8	0.10 ml serum
9	0.10 ml serum

Some litters were divided into three groups: one group received only homologous spleen cells, one group received homologous spleen and normal DBA2 serum, and the third group received homologous spleen cells and DBA2 anti-C57Bl/6 serum.

The results of these experiments are summarized in Table VII and show that the DBA2 anti-C57Bl/6 serum had a marked protective

effect against runting Most of the treated mice survived while most of the controls died of runt disease. The weights of the animals which were treated with DBA2 anti-C57Bl/6 serum were found to be approximately equal to those of normal mice of the same age. In contrast, the weights of mice receiving only homologous spleen cells or homologous spleen cells and normal DBA2 serum were much less than normal for their age.

c) Finally, it was attempted to protect mice from the runting syndrome by immunizing the mothers against the prospective donor strain prior to parturition

One group of experiments was performed by immunizing Balb/C females against C57Bl/6 mice by means of repeated intraperitoneal injections of C57Bl/6 spleen cell suspensions in saline These female mice were mated with normal isologous males and their offspring were treated with C57Bl/6 spleen cell suspensions intraperitoneally within twenty four hours after birth

TABLE VII — *Protection by antiserum against homologous spleen cells*

DBA2 Litter No	*All received C57Bl/6 Spleen Cells on Day of Birth Incidence of Runting*		
	Received Normal DBA2 serum.	*Received DBA2 Anti-C57Bl/6 serum*	*No serum Treatment.*
1	1/2 ( )	0/2	1/1
2	2/2	0/2	
3	1/1	1/2	
4	3/3	1/4	3/3
5	4/4	0/3	2/3
6	1/1	0/2	
7	2/2	3/3	
Total	14/15	5/18	6/7

( ) Numerator indicates number of mice runting: denominator total number of mice in group

The data from this experiment are summarized in Table VIII and indicate that, with certain females, a high degree of protection against runting was observed while with other females, no protection was observed.

A/Jax female mice were immunized against C57Bl/6 mice by repeated inoculation with C57Bl/6 spleen cell suspensions in complete

Freund's adjuvant into the toe pads Offspring of these females with normal A/Jax males received C57Bl/6 spleen cell suspensions intraperitoneally within twenty four hours after birth.

TABLE VIII — *Incidence of runting in litters from mothers immunized against homologous spleen cells*

Treatment of Mothers.	*Breeding Cage*	*Incidence of Runting*
Balb/C mothers immunized against C57Bl/6 spleen cells	1	11/12 (92 %)
	2	3/12 (25 %)
	3	2/10 (20 %)
	4	9/10 (90 %)
Balb/C mothers untreated	—	38/41 (93 %)
A/Jax mothers immunized against C57Bl/6 spleen cells	5 6 & 7	4/20 (20 %)
A/Jax mothers untreated	—	78/79 (99 %)

The data from these experiments, as shown in Table VIII indicate a marked degree of protection against runting in the offspring of immunized mothers. The incidence of runting in these animals was 20 % as compared with a usual incidence of runting of 99 % with this strain combination

Discussion

From our experimental results it can be seen that viable immunologically competent cells (spleen or lymph node) were necessary to produce the runting syndrome Cells killed by repeated freezing and thawing or homogenizing failed to produce runting This finding is comparable to data previously reported by Simonsen [4] that mechanically disrupted cells failed to cause splenic enlargement in newborn chicks. Non immunologically active cells (liver or kidney) did not produce runting It is evident that in order to produce runting the donor cells must be antigenically different from the recipient isologous spleen cells did not produce runting These experiments provide suggestive evidence for the concept of Billingham and Brent [1] and of Simonsen [4] that runting is due to an immunological attack upon the host by living foreign cells given at birth

The decrease in the incidence of runting which was observed to occur as the interval between birth and the time of administration of the foreign cells was increased is reminiscent of the decrease in the

incidence of tolerance in mice observed by Billingham and Brent[1] as they progressively delayed the administration of foreign antigen after birth. They found that tolerance disappeared when the injection of foreign cells was delayed for more than four days. Similarly we observed that, in animals given homologous cells more than four days after birth runting rarely occurred. This suggests that it is the tolerant mouse, in which foreign cells are able to persist and react against the host, that is susceptible to runt disease. Our success in preventing running by treating the mice with isologous adult spleen cells provides further support for this theory. By giving newborn mice isologous adult spleen cells we may well have converted them into an immunologically mature state in which they are able to destroy the foreign spleen cells. Consistent with this hypothesis is the fact that isologous liver cells not being immunologically active, did not confer protection against runting.

In the last two experiments we corrected the immunological immaturity of the newborn mice by providing them with circulating antibody: in one case, by immunization of the mother and reliance upon passive maternal to fetal transfer of antibody, and in the other case, by treating the animals with immune serum. We feel that the success of these methods in protecting against runting suggests the possible involvement of the circulating type of antibody in at least some stage of the immunological reaction by which an animal rejects certain foreign tissues.

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SUMMARY AND CONCLUSIONS

1) Runting was produced by homologous spleen or lymph node cells but not by isologous spleen cells or homologous liver or kidney cells.

2) The incidence of runting (a) varied with the particular strain combination employed (b) increased with larger doses of homologous spleen cells, and (c) decreased as the interval between birth and inoculation with foreign cells increased.

3) Frozen thawed spleen cells did not produce runting.

4) Isologous adult spleen cells protected newborn mice against runting when the isologous spleen cells were given soon after the homologous spleen cells. Isologous spleen cells given one day after birth and isologous liver cells given on the day of birth did not protect against runting.

5) Serum prepared from adult mice immunized against the homologous strain protected newborn recipients of homologous spleen cells from runting

6) Offspring of female mice immunized against the strain to be used as donor were also protected against runting

7) Our data are regarded as compatible with the concept presented by previous workers, that runting is an immunological reaction of foreign cells against a tolerant host. Our data further emphasize the possible importance of circulating type of antibody in the immunological reaction by which an animal destroys certain foreign tissue

DISCUSSION

GORER One reference would interest Dr Siskind and that is some work done by a Czech scientist Dr Zekla using the Walker rat carcinoma. He found he could get passive protection by serum in the ordinary way and he also showed that this protection could be transferred in the milk which is rather like the result just reported Now there is one thing I would like to say about the use of adjuvants which seem to be extremely fashionable these days. It is possible that by putting them in the footpad you may get more effect dose for dose than if you put the same dose of spleen cells in the peritoneum without the adjuvant That I do not know but in work at this stage of homograft studies I think adjuvants are theoretically undesirable, because you may be producing something the animal would not produce in the ordinary way with a homograft We have tried adjuvants and we have found that you get adequate titres without them We have not used the footpad because we find that we cannot get what we call adequate doses into the footpad and I think that Dr Siskind would probably have got much clearer results if he had given larger doses of cells intraperitoneally without adjuvant That is a practical point which I think is well worth thinking about.

SISKIND We did do one group of experiments on Balb/c mice in which we did immunise intraperitoneally without adjuvant We had results that were essentially similar to our other work except that there was less consistency from mouse to mouse than when we had used adjuvants. It was after observing this lack of consistency without adjuvants that we began to use adjuvants in our work

KNAKE I do not want to object to your results Dr Siskind but I doubt whether the lesions in the liver are a syndrome of runt disease I think they look a little like paratyphoid lesions Are you sure that these lesions in the liver were not caused by infection ?

SISKIND We have attempted by several procedures to rule out the possibility of infection. We find that various procedures, such as homogenizing the spleen cells, freezing and thawing the spleen cell suspensions grinding up cell suspensions and then centrifuging and using the cell free supernatant, which destroy cells abolish the ability of the preparation to produce both the runt disease as such and the liver lesions The procedure mentioned would not, I think be expected to destroy most infectious agents. The liver lesion is seen quite uniformly in runted mice. It is not seen if you put isologous tissue into newborn animals. The histology of the lesion is consistent with what one might expect from some sort of thrombotic phenomenon. We do not feel that an infectious agent *per se* is responsible for the runt phenomenon. However it is possible that in a debilitated mouse infections might occur and these might account for the liver lesions observed

OWEN I have two questions one of which is related to Dr Gorer's previous reference to milk transmission. I wondered whether you had done any fostering experiments with the progeny of the immunized females. Such experiments would be interesting in both directions that is, whether young born to these females before nursing retained their resistance, and whether young born to an untreated female but fostered to an immunized female might acquire resistance by way of her milk The second question is whether you have any evidence of a correlation between the runting syndrome and the tolerant state that is, when your treated mice resist runting do they nevertheless acquire tolerance ?

SISKIND In answer to the first question I quite agree that fostering experiments would be very interesting We have not done this because we were anxious to demonstrate the existence of protection and so designed the experiment as to have the maximum opportunity to demonstrate protection We thought that leaving the newborn mice with their own *mother* would give the best chance of demonstrating protection and would introduce the fewest possible extraneous variables With regard to your question as to the association between runt disease and tolerance In the system with which we work we see very few mice living more than 20 days. We do not have an opportunity to graft our mice, and so we really have no data on tolerance We can only say that the time relationships for obtaining runt disease and for obtaining tolerance are very similar That is our incidence of runting decreases as you increase the interval between birth and the injection of foreign cells in a manner similar to that reported by Billingham Brent and Medawar for the decrease in the incidence of tolerance

ALBERT First of all I would congratulate Dr Siskind on his nice imunication and his splendid presentation May I ask Dr Siskind reason why when he injects the isologous cells one day after birth no longer has the same effect ?

SISKIND This result is very striking We have no good explanation it Apparently something very important happens quite quickly after this critical period we are no longer able to protect. We can explain this. We can only say that perhaps the foreign cells once have established themselves now have obtained some sort of pri ged position. Also there is probably a great increase in the number oreign cells. Perhaps some sort of privileged position of the foreign which are present in large numbers might be involved

ALBERT By injecting homologous cells from an adult donor into mmunologically immature recipient one obtains simultaneously tole e and runt disease. Both phenomena seem to depend on the sur l and the proliferation of these homologous cells in the organism he host.

How does it happen then that on injecting a tolerant recipient with phoid cells of its own strain, one can suppress this acquired tole e even weeks or months later whereas one day after birth you are longer capable of avoiding runt disease ? Must one conclude that e is a difference between the mechanism of tolerance induction and of runt disease ?

SISKIND When *Billingham* abolishes *tolerance with lymph node* long after its production it takes fifteen to thirty days before the rated graft is rejected We do not have that length of time since animals die by 15 days after birth So it is perhaps just a problem iming Perhaps if instead of using normal adult isologous cells we l immune adult isologous cells, we might still be able to protect these nals one day after birth We are in the process of trying this now we do not have any data on it yet.

MEDAWAR May I make just one comment in order to clarify the ition of the relationship between tolerance and runt disease ? The iction of tolerance is a *necessary* condition for the production of disease when one is injecting embryos but there is no other neces connection between the two phenomena One can get tolerance out runt disease by three possible methods. The first is to induce rance with embryonic cells the second is to use F_1 hybrid cells

injected into parental newborns and the third is to use adult cells which are not immunologically competent for example monocytes. The fact that under these three circumstances one can get tolerance without runt disease shows that there is no necessary causal connection between them

SIMONSEN I would like to congratulate Dr Siskind on this very nice piece of work. He has in fact answered the most important of the questions I wanted to ask—the transplantation of isologous cells which have been pre-immunised against the donor But there are a few more questions I would like to put. Did you look at your spleens in those runts which died after some 20 or 30 days to see if they were enlarged or atrophied ? Did you ever observe ascites in the early deaths you got after homologous spleen cells ? You showed these liver necroses which I have also seen repeatedly but only I think if I transplanted to homozygous recipients If I transplanted to F_1 hybrids, they did not occur which I think is a gene dose phenomenon. But sometimes I have seen ascites developing in the homozygous recipients apart from liver necroses.

SISKIND The spleens in our runted mice were quite variable in size. Most of them appeared to be slightly enlarged and pale in colour There was an occasional small one, but, as I said the vast majority were large for the size of the mouse. We did not usually see ascites, although there were a few questionable cases.

BRENT I think that I can throw some light on the question of whether tolerance is necessary for the occurrence of runt disease. I do not think there is any question that tolerance can occur without runt disease, as experiments with hybrid mice and other kinds of experiments have shown But although it is true to say that tolerance usually aids and abets runt disease—that it may frequently be a necessary prerequisite for the occurrence of a graft versus-host reaction it does not necessarily follow that this is always so It is sometimes possible to hit on a strain combination in which runt disease can occur in the almost complete absence of tolerance. For example experiments carried out by Billingham and myself have shown that adult *AU* strain spleen cells intravenously injected to *A* strain newborns will invariably cause the death of the recipients Here then is clear-cut evidence of acute runt disease However the mortality can be circumvented by the injection of either AU bone marrow cells or *AU/A* F_1 hybrid spleen cells but neither of these cell types has been found to elicit good tolerance of AU skin grafts. The AU spleen cells therefore appear to be able

ALBERT First of all I would congratulate Dr Siskind on his nice communication and his *splendid presentation. May I ask Dr Siskind* the reason why when he injects the isologous cells one day after birth he no longer has the same effect ?

SISKIND This result is very striking We have no good explanation for it. Apparently something very important happens quite quickly and after this critical period we are no longer able to protect. We cannot explain this. We can only say that perhaps the foreign cells, once they have established themselves, now have obtained some sort of privileged position Also there is probably a great increase in the number of foreign cells. Perhaps some sort of privileged position of the foreign cells which are present in large numbers might be involved.

ALBERT By injecting homologous cells from an adult donor into an immunologically immature recipient, one obtains simultaneously tolerance and runt disease. Both phenomena seem to depend on the survival and the proliferation of these homologous cells in the organism of the host.

How does it happen then that on injecting a tolerant recipient with lymphoid cells of its own strain one can suppress this acquired tolerance, even weeks or months later whereas one day after birth you are no longer capable of avoiding runt disease ? Must one conclude that there is a difference between the mechanism of tolerance induction and that of runt disease ?

SISKIND When Billingham abolishes tolerance with lymph node cells long after its production it takes *fifteen to thirty days before the* tolerated graft is rejected We do not have that length of time since our animals die by 15 days after birth So it is perhaps just a problem of timing Perhaps if instead of using normal adult isologous cells we used immune adult isologous cells, we might still be able to protect these animals one day after birth We are in the process of trying this now but we do not have any data on it yet.

MEDAWAR May I make just one comment in order to clarify the question of the relationship between tolerance and runt disease ? The induction of tolerance is a *necessary* condition for the production of runt disease when one is injecting embryos but there is no other necessary connection between the two phenomena One can get tolerance without runt disease by three possible methods The first is to induce tolerance with embryonic cells the second is to use F_1 hybrid cells

injected into parental newborns and the third is to use adult cells which are not immunologically competent for example monocytes The fact that under these three circumstances one can get tolerance without runt disease shows that there is no necessary causal connection between them

Simonsen I would like to congratulate Dr Siskind on this very nice piece of work. He has in fact answered the most important of the questions I wanted to ask—the transplantation of isologous cells which have been pre-immunised against the donor But there are a few more questions I would like to put. Did you look at your spleens in those runts which died after some 20 or 30 days to see if they were enlarged or atrophied ? Did you ever observe ascites in the early deaths you got after homologous spleen cells ? You showed these liver necroses which I have also seen repeatedly but only I think if I transplanted to homozygous recipients. If I transplanted to F_1 hybrids, they did not occur, which I think is a gene dose phenomenon. But sometimes I have seen ascites developing in the homozygous recipients apart from liver necroses.

Siskind The spleens in our runted mice were quite variable in size. Most of them appeared to be slightly enlarged and pale in colour There was an occasional small one, but, as I said the vast majority were large for the size of the mouse. We did not usually see ascites although there were a few questionable cases.

Brent I think that I can throw some light on the question of whether tolerance is necessary for the occurrence of runt disease. I do not think there is any question that tolerance can occur without runt disease, as experiments with hybrid mice and other kinds of experiments have shown. But although it is true to say that tolerance usually aids and abets runt disease—that it may frequently be a necessary pre requisite for the occurrence of a graft versus-host reaction it does not necessarily follow that this is always so It is sometimes possible to hit on a strain combination in which runt disease can occur in the almost complete absence of tolerance. For example, experiments carried out by Billingham and myself have shown that adult AU strain spleen cells intravenously injected to A strain newborns will invariably cause the death of the recipients. Here then is clear-cut evidence of acute runt disease However the mortality can be circumvented by the injection of either AU bone marrow cells or AU/A F_1 hybrid spleen cells, but neither of these cell types has been found to elicit good tolerance of AU skin grafts. The AU spleen cells therefore appear to be able to

kill their hosts either without having induced tolerance at all or in the presence of a tolerance which is far from complete. Our inference is that the reaction of the AU cells against the A strain host antigens is so prompt and violent that they can kill the hosts before the relatively immature hosts have been able to react against them. May I add that Dr Siskind's interesting experiments are very much in agreement with the work done by Billingham and myself? We have also shown that the concomitant injection of adult isologous cells will protect the recipients from the otherwise lethal effect of homologous spleen cells. In addition we have found that the pre-sensitisation of adult homologous cells injected into neonatal animals will increase the incidence and severity of runt disease, further showing that the disease is due to an immunological reaction of the injected cells against the tissues of the hosts.

BETZ I would also like to comment on the liver lesions in those animals. I agree with Dr Knake they look like infectious lesions. We found quite similar liver lesions in irradiated animals. In mice irradiated with two doses of X rays, we find liver lesions in animals dying around the 9th or 10th day and when we find them they are always in animals with a very low level of leucocytes and also with a bacteraemia. In these animals, we noticed the presence of bacteria in the blood and the spleen. As we discovered liver lesions later on we did not search for bacteria in the liver itself. But now we are doing new experiments to check this. I would like to ask you if you have an idea about the existence of a bacteraemia in your animals during the last days of life because those lesions must be very acute and they appear surely shortly before death.

SISKIND We have no evidence for or against a bacteraemia towards the terminus of these animals. We are not convinced that these lesions are of an acute nature. We can kill runted animals at any time after about 9 days of age and see lesions of this sort. They do not seem to change if you take the sections earlier or later in the disease. We do not know what the pathogenesis is of these lesions but if they should turn out to be infectious it would not be surprising to see an infection in an animal which is as sick as these animals are.

KLEIN I was also very interested Dr Siskind in your finding that immunised mothers can transmit something that reduces the incidence of runting in the offspring and I wonder whether the antibodies transmitted are powerful and competent enough to produce the opposite result under given circumstances. Have you tried to cross your immunised mothers with males which you have used for the immunisation to see

whether there is any evidence of running in the F_1 or any other abnormality ?

SISKIND We have not attempted to do this sort of procedure. It would be interesting. We have just recently given serum immunised against the recipient to newborn mice and in a few cases we have observed various types of lesions. The lesions which we have observed are not the same as those we observe in the running phenomenon. We see a focal necrotic glomerular lesion and a lesion in the liver which is also focal necrotic, but looks somewhat different from the one you see with running. We do not know whether these lesions are the result of antibody antigen reactions whether they are the results of some non-specific effects or whether they are the result of haemagglutination resulting in thrombotic phenomena. The possibility of a thrombotic phenomenon is the most entertaining because the lesions look histologically as if they might be of thrombotic origin. We have not seen these lesions in control animals receiving normal serum only in animals receiving serum immunised against the recipient. However the number of animals we have worked on with this procedure is too small to be certain of the results.

VOISIN I feel compelled to say a few words about the work done by Dr Louise Leonard who did her doctorate in Dr Thomas's laboratory and who is at the present time working in my laboratory. While she started working on acquired tolerance, she got very little tolerance but a very high percentage of running so she also turned to the problem of runt disease. And in many respects she had the same experience as Dr Siskind. That is to say she observed the same specific liver lesions and felt that the interpretation of thrombosis either by haemagglutination or by the reaction of the injected cells towards the endothelium of the host seemed to be the most satisfactory. Furthermore she was also able to protect the host by simultaneously injecting isologous cells. This and several other original experiments form the basis for her Ph. D thesis.

SISKIND One of the other experiments which Dr Leonard did which is of interest is the transfer of running. I believe that she took spleens from runted animals injected them into newborn isologous animals and observed running with the usual liver lesions.

VOISIN That is correct and I think we can even go further and say that she got the same results taking the spleens of tolerant animals

BRENT Dr Siskind will probably agree that it is very difficult to be certain of the exact cause of death in these animals We know that their condition is ultimately caused by a graft versus-host reaction, but we do not know at present what the *immediate* cause of death is. It may well be that they are dying from secondary infections arising from their general debilitation but it is very difficult to be certain of that As Dr Siskind we too have ruled out the possibility of infection as the *primary* cause of runt disease

SISKIND We also have been much interested in what causes the death of these animals and recently we have been examining histologically just about any tissues we can obtain from these animals. So far outside the liver lesions and certain changes in the blood picture we have been unable to discover any lesions. With regard to the blood picture we find and I think that Dr Leonard also found an anemia of very variable degree and a marked variability in the white blood cell count with counts which ranged all the way from a striking leucopenia to a very marked leucocytosis

Some reactions observed with transplanted reticulo endothelial cells in mice

P A. GORER and E. A BOYSE.

(Department of Pathology Guy's Hospital Medical School, London S.E. 1)

Although the experiments to be described here are concerned chiefly with normal reticulo-endothelial cells they were undertaken to throw light upon previous experiments with leukaemias.

Like all neoplasms leukaemias tend to become less exacting in their growth requirements with succeeding transfers—the rate at which these changes occur showing considerable variation from case to case. A few leukaemias will grow as sub-cutaneous grafts from their earliest transfers but the majority will not do so even as isologous grafts until they have experienced several transfers within their strain of origin. Until a leukaemia will give palpable subcutaneous growths it is useless for work on homografts and thus most of our knowledge is based upon cases that have been transferred for some years.

Potter and Findley [11] first showed that the response to an incompatible leukaemic graft differed from that found with other types of growth We have since confirmed this with other leukaemias [8, 9] Destruction of all other types of homografts in unimmunised hosts is intimately associated with cellular invasion from the host With leukaemias however destruction follows massive exudation of plasma and the hosts cells usually intervene relatively late—at least in subcutaneous homografts. The observations of Weaver [21] suggest that the course of events may be somewhat different in the peritoneal cavity Thus all the anatomical findings support the view that leukaemic cells are susceptible to direct attack by antibody This deduction is strongly supported by a great deal of experimental evidence some of which will be briefly summarised

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Gorer and Amos [11] demonstrated passive immunity to a C57Bl leukosis (E.L.4) not only in three foreign strains (A BALB/c and C3H)

but also in the C57BL strain itself. Amos and Day[7] extended these observations to three other leukaemias in three different pure lines. The antigens and antibodies responsible for protection in the strain of origin were called « X » and « anti X » respectively. Amos and Day found that all four X components which they studied were antigenically distinct. Unpublished work in this laboratory indicates that leukoses arising in the same sub-strain of C57BL have related X components which are distinct from those occurring in another sub-strain of C57BL. The question naturally arises whether the X antibodies are responsible for the peculiar susceptibility of leukaemias; unfortunately this does not appear to be so. Gorer and Amos found that sera produced against a C57BL mammary carcinoma or against normal C57BL tissue, although containing no detectible anti X, would protect against homografts. Amos and Day found that anti X was associated with the alpha and beta globulins while the ordinary isoantibodies were associated with the gamma globulins. The former would protect any strain of mice, the latter would protect only against homografts.

The fact that serum may give passive protection against bacterial infection is no proof of direct bactericidal action *in vivo*; indeed in many infections it is the cytotropic and opsonising properties of antibodies that are of major importance. However Amos and Wakefield[8, 9] have shown that leukaemic cells may be destroyed in Algire-type diffusion chambers in actively immunised or passively immunised homologous hosts. We have recently confirmed this using a different leukaemia (Figures 1a to 1c). No data are as yet available on the action of anti X within chambers. Unfortunately anti X is apparently not cytotoxic *in vitro*.

While it seemed clear that anti X is not solely responsible for the high susceptibility of leukotic cells to antibody, it remained to be determined whether this susceptibility could be specifically associated with this particular variety of malignancy or whether it was inherited from the normal precursors of the malignant cell. This problem was investigated by experiments *in vitro* and *in vivo*.

Since the pioneer work of Ritz in 1911 it has been known that mouse sera seldom show complementary activity *in vitro*, although the results in diffusion chambers by Algire and colleagues[3], Amos and Wakefield and ourselves show that complementary activity is effective *in vivo*. For tests *in vitro* O'Gorman and the senior author[11] used guinea pig or rat complement. We found that the A strain sarcoma Sa I was almost completely resistant to cytotoxic action and that the C3H sarcoma BP8 was apparently a heterogeneous population in this respect. All of five leukaemias were susceptible although two recent

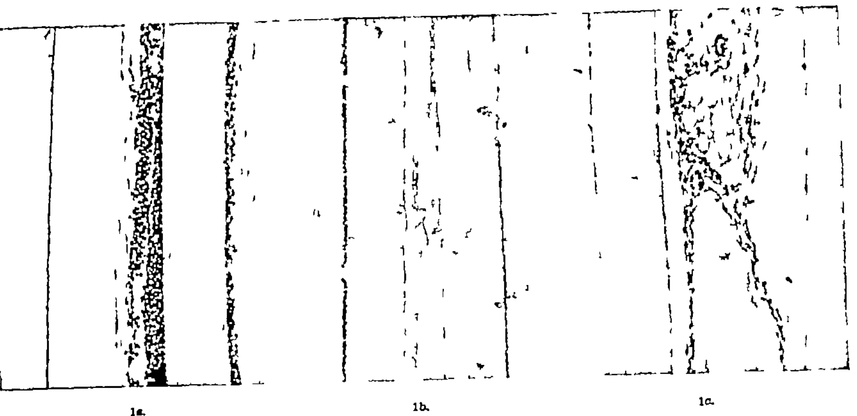


FIG. 1a. — Cells from sub-cutaneous growth of BALB/c leukaemia on seventh day in diffusion chamber in unimmunised A strain mouse

FIG. 1b — Destruction of cells BALB/c leukaemia on seventh day in a chamber in immunised A strain mouse.

FIG. 1c. — Another part of same chamber as in previous figure. Note persistence of BALB/c fat and fibrous tissue in spite of destruction of leukaemia cells.

ones E.L.5 and E.L.8 were also highly susceptible to the cytotoxic action of unheated guinea pig serum. Normal cells from spleen and lymph nodes behaved like the longer-established leukaemias.

We are at present extending these studies to thymus and bone marrow. Preliminary results indicate that many thymus samples are highly sensitive to the cytotoxic activity of guinea pig serum and to a lesser extent rat serum, like two of the recent leukaemias. Unfortunately these two leukaemias were not tested against rat serum during their early transfers and they have now lost their sensitivity to guinea pig serum. The earlier behaviour of E.L.5 and E.L.8 is of some interest. The former was diagnosed as a thymoma at autopsy whilst the latter mouse had generalised lesions and a greatly enlarged thymus and it is likely that the thymus was the primary focus.

The reactions of thymus to antibody have been variable (Table I). The results obtained with the nodes of the one month-old BALB/c mouse is what might be expected from a good serum giving 95-100 per cent kill at the dilutions stated. The thymus appears to be composed almost entirely of antibody resistant cells. On the other hand the thymic cells of the young C57BL, exposed to a different and less potent serum are slightly more sensitive than lymph node cells. It will be noticed that this serum shows some zoning; this is not uncommon and may be more extreme than in this example.

TABLE I

A comparison of Thymus and Lymph nodes from 1 month old mice

	Strain of mouse	Tissue	C C	Percentage cells killed by serum diluted one in 8	16
C57 anti B c[1]	B c	L. nodes	7	100	95
C57 anti B c	B c	Thymus	8	19	20
A anti C57	C57	L. nodes	7.8	75	78
A anti C57	C57	Thymus. Rat complement was used	7.0	72	93

[1]C57 = C57BL [2]B c = BALB c [3]C C = complement control

Bone marrow suspensions are nearly always appreciably less susceptible than lymph nodes and spleen. It may be that the variations between different samples of bone marrow or thymus reflect the func

tional state of the organs But the picture is complicated by factors such as the anti-complementary properties of various cell suspensions. That this may be important is illustrated by the observation that the washing of one cell suspension increased the number of cells killed by antibody from 70 per cent (unwashed) to 98 per cent (washed) The complement controls were not significantly different.

These findings certainly strengthened the impression that the susceptibility of leucotic cells is an inheritance from their normal precursors and we wished to confirm this by experiments *in vivo*

It is not possible to study homografts of normal reticulo-endothelial cells in quite the same way as with leukaemias as they do not exhibit similar proliferation in the sub-cutaneous space. We therefore tried two other methods of approach In the first of these we studied the destructive effect of antibody upon homologous marrow or splenic cells administered to protect mice exposed to lethal whole-body irradiation (850-900 r).

In most experiments we used A strain hosts and marrow or spleen from C3H BALB/c or C57BL donors. In others we used BALB/c hosts and C57BL or (BALB/c × C57BL) F_1 donors. The design of these experiments was very simple. In all of them two groups of irradiated mice were used and both were given homologous marrow or spleen intravenously One group also received 0.2 0.25 ml of immune serum directed against the injected homologous cells, the other (control) group received either saline or normal mouse serum. An additional group of irradiated unprotected mice was sometimes included these received only the streptomycin which was given to all mice.

We used hyperimmune sera against various neoplasms. All were titrated according to our routine haemagglutinin or cytotoxic techniques[13, 14] The latter is more useful here because A anti-C3H and A anti BALB/c usually show no haemagglutinating activity The cytotoxic titres were usually of the order of 128 when tested with splenic cells. Decisive results were obtained with marrow cell doses of 3-6 million per mouse. The protection of the control groups receiving these doses was entirely adequate whereas the experimental animals all behaved as if the serum had completely abolished this protection and they died like the irradiated unprotected mice.

Table IIa shows the results of two experiments with marrow and one with spleen. As a further control on the specificity of the serum reactions we undertook the experiments in Table IIb using the same antisera. Again we used two groups of irradiated mice but in this instance one group received isologous marrow and the other homologous marrow and *all* mice of *both* groups received the same antiserum As

the table shows the former survived and the latter died as before Thus there is no evidence of any anti-organ antibody or any toxic element in the serum other than specific isoantibody

TABLE II a

The lethal effect of antibody on transfused homologous marrow or spleen in irradiated mice

Tissue	Host	Donor	Type of serum	Proportion surviving on days following irradiation							
				4	5	6	7	8	9	10	11
B.M.	A	B/c	N	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
B.M.	A	B/c	Imm	8/8	8/8	8/8	6/8	4/8	1/8	1/8	0/8
B.M.	B/c	C57	N	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
B.M.	B/c	C57	Imm	7/8	7/8	4/8	0/8	0/8			
Spl	B/c	(C57 × B/c)	N	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Spl	B/c	(C57 × B/c)	Imm	5/5	5/5	5/5	4/5	1/5	1/5	1/5	1/5

Note B.M. = bone marrow Spl = spleen B/c = BALB/c C57 = C57BL N = normal serum Imm = immune serum In Expt 1 A anti BALB/c Expt. 2 and 3 BALB/c anti C57BL

TABLE II b

Specificity of Cytotoxic Effect of Immune Serum on Bone Marrow cells

Host	Donor	Serum	Proportion surviving on day following irradiation				
			7	8	9	10	11
A	B/c	Imm	5/5	1/5	0/5	—	—
A	A	Imm	5/5	5/5	5/5	5/5	5/5
B/c	C57	Imm	5/5	5/5	2/5	1/5	0/5
B/c	B/c	Imm	5/5	5/5	5/5	5/5	5/5

The immune sera were the same samples of A anti BALB/c and BALB/c anti C57BL as shown in Table IIa

It was usually possible to distinguish between the appearance of protected mice and those in which protection had been abolished on the fourth or fifth days after irradiation In one experiment with spleen it was clear by the seventh day that cell dosage had [illegible] inadequate and all survivors were killed. A distinct difference w[illegible] between

the histological appearances of the serum and control groups. The former resembled untreated mice but the spleens of the latter showed extensive repopulation.

The BALB/c mouse in Table IIa that received 13·7 million F_1 spleen cells was our only survivor in any serum group. It was in poor health and was therefore killed on the 62nd day for examination of its spleen and red cells. These were typed in order to see if by any chance there had been regeneration of the host's R.E. system. This was not so: the reactions were those of a (BALB/c × C57BL) F_1 mouse. In general information obtained with the cytotoxic test could have been anticipated from the data yielded by other methods but a few points are of interest. The earliest test on a chimaera was made on the eleventh day. Here the spleen gave the sort of titration one normally expects from marrow. The spleen of a chimaera showing definite signs of secondary disease and tested on the sixtieth day appeared to contain little but primitive cells. These were not unduly sensitive to guinea pig serum but were highly susceptible to antibody and 100 per cent kill was obtained in several tubes. Thus all free cells were apparently of a donor origin.

Tests of this kind have the advantages that they are quick and do not depend upon the presence of dividing cells. No constant figures can be given to indicate the sensitivity of the tests in the detection of mixed cell populations because this must depend upon the particular sensitivity to antibody of the cells under examination, the relative ease with which various cell types can be dispersed into the suspending fluid and the proportion of dead cells present in control tubes. With radiation chimaeras our tests on spleen have been clear cut. For example, we have injected mixtures of cells from different F_1 mice with one parental strain in common into mice of the shared pure line and in some of these experiments survival was good. All their spleens were later found to behave as if of one donor F_1 type only. Apparently one partner had destroyed the other without adverse effect upon the host.

The fact that serum treated mice die as rapidly as untreated controls indicates that the essential cell types have been destroyed. We do not know how many types of cells must be present simultaneously for protection against radiation death. It is possible that the proportion of cells that need to be destroyed before protection is lost is quite small. The maximum number of cells destroyed by 0.2 ml of one sample of serum may really be much smaller than the 13–14 million suggested by Table IIa. However this may be it seems certain that undifferentiated cells play a vital part and the evidence suggests that most of them must be destroyed. Furthermore it also seems virtually certain that leukae

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Spl	B/c	(C57 × B/c)	Imm	5/5	5/5	5/5	4/5	1/5	1/5	1/5	1/5

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A	B/c	Imm	5/5	1/5	0/5	—	—
A	A	Imm	5/5	5/5	5/5	5/5	5/5
B	C57	Imm	5/5	3/5	2/5	1/5	0/5
B	B/c	Imm	5/5	5/5	5/5	5/5	5/5

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B.M.	B/c	C57	N	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
B.M.	B/c	C57	Imm	7/8	7/8	4/8	0/8	0/8			
Spl	B/c	(C57 × B/c)	N	5/5	5/5	5/5	5/5	5 5	5/5	5/5	5/5
Spl	B/c	(C57 × B/c)	Imm	5/5	5/5	5/5	4/5	1/5	1/5	1/5	1/5

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A	A	Imm	5 5	5, 5	5 5	5, 5	5/5
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B c	B c	Imm	5 5	5/5	5/5	5 5	5/5

The immune sera were the same samples of A anti-BALB/c and BALB/c anti C57BL as shown in Table IIa

It was usually possible to distinguish between the appearance of protected mice and those in which protection had been abolished on the fourth or fifth days after irradiation. In one experiment with spleen it was clear by the seventh day that cell dosage had been inadequate and all survivors were killed A distinct difference was found between

the histological appearances of the serum and control groups. The former resembled untreated mice but the spleens of the latter showed extensive repopulation.

The BALB/c mouse in Table IIa that received 13.7 million F_1 spleen cells was our only survivor in any serum group. It was in poor health and was therefore killed on the 62nd day for examination of its spleen and red cells. These were typed in order to see if by any chance there had been regeneration of the host's R.E. system. This was not so: the reactions were those of a (BALB/c × C57BL) F_1 mouse. In general information obtained with the cytotoxic test could have been anticipated from the data yielded by other methods but a few points are of interest. The earliest test on a chimaera was made on the eleventh day. Here the spleen gave the sort of titration one normally expects from marrow. The spleen of a chimaera showing definite signs of secondary disease and tested on the sixtieth day appeared to contain little but primitive cells. These were not unduly sensitive to guinea pig serum but were highly susceptible to antibody and 100 per cent kill was obtained in several tubes. Thus all free cells were apparently of a donor origin.

Tests of this kind have the advantages that they are quick and do not depend upon the presence of dividing cells. No constant figures can be given to indicate the sensitivity of the tests in the detection of mixed cell populations because this must depend upon the particular sensitivity to antibody of the cells under examination, the relative ease with which various cell types can be dispersed into the suspending fluid and the proportion of dead cells present in control tubes. With radiation chimaeras our tests on spleen have been clear cut. For example, we have injected mixtures of cells from different F_1 mice with one parental strain in common into mice of the shared pure line and in some of these experiments survival was good. All their spleens were later found to behave as if of one donor F_1 type only. Apparently one partner had destroyed the other without adverse effect upon the host.

The fact that serum treated mice die as rapidly as untreated controls indicates that the essential cell types have been destroyed. We do not know how many types of cells must be present simultaneously for protection against radiation death. It is possible that the proportion of cells that need to be destroyed before protection is lost is quite small. The maximum number of cells destroyed by 0.2 ml of one sample of serum may really be much smaller than the 13–14 million suggested by Table IIa. However this may be, it seems certain that undifferentiated cells play a vital part and the evidence suggests that most of them must be destroyed. Furthermore it also seems virtually certain that leukae-

mic cells retain many of the immunological properties of their precursors.

The ability to form circulating antibody is an alternative method by which to study the survival of R.E. cells after transfer to another individual. Here we are more confident of the essential cell lineage. Harris, Harris and Farber[14] used this method to show that lymph node cells in rabbits behave like murine leukotic cells. They found that lymph node cells exposed in vitro to a filtrate prepared from a trypsinised suspension of Sh. paradysenteriae would produce agglutinins when transferred to other rabbits. But if the recipients had been pretreated with leukocytes from the prospective donors the formation of agglutinins was suppressed. An interesting feature of this work was the rapidity with which signs of active immunity developed. Some immunity was evident as early as the second day, was fully developed by the sixth day and had not completely declined by the hundredth day. Subsequently they showed that this immunity could be transferred by immune serum and by some normal sera.

Mitchison[14] made some interesting observations on the production of antibodies to E. typhosum by splenic cells in homologous hosts. The difference in survival times in immune and non-immune hosts was not very striking and therefore one of us (Boyse[4]) attempted a modified approach. Human or sheep red cells were used as antigen. Suspensions of washed splenic cells from hyperimmune donors were transplanted intra-peritoneally. In this system there was little output of saline haemagglutinins in the hosts unless they received a challenge dose of antigen. The critical challenge dose of red cells gave a minimal primary response but a good secondary response (of the order of 1000). Isologous grafts were shown to function for as long as 46 days while homologous grafts gave no evidence of function, not even when the hosts were challenged as soon as 30 hours after transplantation. Apparently this test was too exacting for the original purpose of demonstrating passive immunity to transplanted R.E. cells.

The work of Medawar and his colleagues has focussed attention on the importance of cellular sensitisation in homograft reactions. It seemed to us that the reactions of parental line R.E. cells in an F_1 host might throw some light on this aspect of the problem. The combination studied in most detail was (A × C57BL) F_1. It was possible to demonstrate survival of A strain splenic cells for as long as 46 days in the F_1 although the results were not so consistent as with the isologous (A into A) controls. Function of C57BL splenic cells in the F_1 appeared to fail after fourteen days at the most.

A strain cells from donors immunised against sheep red cells produced high sheep haemagglutinin titres in the absence of specific challenge. If the donors had been immunised also against C57BL tissue the output of sheep (or human) haemagglutinins was greater still. No such titres were obtained from transplants of C57BL cells. F_1 hosts receiving iso-immune A strain transplants developed symptoms not unlike secondary disease and died on the 19th–28th days. F_1 hosts receiving comparable transplants from iso-immune C57BL donors showed no ill effects. As revealed by the secondary response to the human red cell antigen these C57BL transplants were functional for 1–2 weeks but there was a definite indication that iso-immunisation of the donors (in addition to immunisation against heterologous red cells) accelerated their disappearance in the F_1.

We have studied the pathological effects of iso-immune A strain splenic cells, lymph node cells and thymic cells. The effective dosage may be lower than 20 million cells per mouse which is incomparably less than that used by Castermans[7] in homologous transplants with somewhat similar results. Lymph nodes cells are probably more rapidly lethal than splenic cells. No effect has yet been seen with thymic cells.

The lesions are partly dependant upon the route of inoculation. Following intra-peritoneal inoculation there is a massive invasion of the pancreas by small mononuclear cells with considerable destruction of the parenchyma together with massive fat necrosis, a picture reminiscent of acute pancreatitis in man. Blood stained ascites develops and the mice become emaciated and die within four weeks. Following intravenous inoculation pancreatic lesions are of no importance. In both instances the spleen and lymph nodes appear to be replaced by graft histiocytes. Histological evidence of this replacement is first apparent by the sixth day and is fairly complete by the tenth day. The cytotoxic test confirms the histological appearances. On the fourth day the spleen behaves as an F_1 spleen, on the sixth day as a mixed population and from the tenth to fourteenth day as an A strain spleen. Some mice may die about the 28th day but others may live for very much longer. Red cell typing shows that in the latter group the marrow is gradually replaced and may become an A strain marrow.

Iso-immune C57BL splenic cells in the (A × C57BL) F_1 may show initial evidence of colonisation but they appear to die out in the spleen as indicated in the experiments with the production of red cells agglutinins. However if inoculated into the (BALB/c × C57BL) F_1 a chronic illness may be produced and one such mouse examined had a C57BL spleen and marrow. As so often happens in work of this kind results vary with the donor–host combinations studied.[12]

There would appear to be two possible explanations of the destruction of C57BL cells in the F_1 with the A strain. The F_1 may lack some antigen present in the C57BL. This could occur through more than one genetic situation the simplest of which is a *completely recessive* antigen in the C57BL stock. No isoantigen has yet been shown to be recessive in the sense that heterozygotes have been shown able to react against homozygotes. It is very well known that some blood group antigens give relatively weak reactions when heterozygous, the most notorious example being the human A_2B which was often erroneously diagnosed as group B in the past. Presumably this so-called dosage effect is *due to the fact that* less antigen is formed in the heterozygote and it does not seem impossible that no antigen is produced in some cases. If such antigens exist in mice as suggested by Simonsen et al. [19] they must certainly be very weak or very rare. If they are very weak a very exacting test such as that of Boyse [3] might be needed to reveal their presence. *The fact that C57BL splenic cells* can survive in the F_1 with BALB/c may indicate that these two strains share the antigen in question. On the other hand there are grounds for thinking that destruction may be allergic, i.e. consequent upon the reaction between antigen and sessile antibody. Experiments to decide between these alternatives have not *yet been completed (December 1958).*

Whatever may be the explanation of the destruction of C57BL cells in the cross with the *A* strain there are grounds for believing that *allergic destruction* of lymphocytes and their allies plays an important part in the various manifestations of graft versus host diseases. Cytolysis is normally brought about by free antibodies directed against antigens that form an integral part of the cells or adsorbed upon their surfaces. However the antibody may be fixed to the cells and the antigen present in the environment. Fink Smith and Rotlauf [8] claimed to have demonstrated a reaction of the latter type in cells in the peritoneal exudate of mice treated *with lyophilised tumour and Freund's adjuvant.*

In mice three varieties of « graft » disease have been described runt disease of Billingham and Brent [4, 5] secondary irradiation disease and that described here (which is essentially the same as that of Castermans [7]). Secondary irradiation sickness differs in various ways from the other *two but all result in a gross deficiency of lymphocytes* and both irradiated mice and F_1 mice show a hunched appearance and wasting. It has been suggested that depletion of the lymphocytic elements is due to exhaustion. However these elements are not particularly easily exhausted in chronic infections or in animals used for the production of antitoxins, etc. In some F_1 mice an infective hepatitis develops. The spleens of such animals are still quite capable of myelopoiesis. Furthermore the

virtual disappearance of lymphoid elements proceeds extremely rapidly in F_1 disease and in runt disease. Lastly the usual consequence of excessive antigenic stimulation is not atrophy by exhaustion but immunological paralysis. It therefore seems a reasonable deduction that these elements do not survive because they are susceptible to allergic destruction It is noteworthy that the most lethal donor for new borne mice was C57BL. No survivors were found amongst infant A strain or CBA strain mice so treated and only one in strain AU The C57BL strain exists in many sub-strains most of which have the H-2b antigens whilst some have H-2d There is no evidence that any sub-strain destroys normal or neoplastic homografts with peculiar efficiency Their lethality as donors may therefore be due to their susceptibility to allergic destruction rather than peculiar powers of aggression. It remains to be seen how these speculations survive but is evident that Medawar's analogy between homograft reactions and tuberculin type allergy may help us to understand more than the rejection of an orthotopic skin graft.

SUMMARY AND CONCLUSIONS

Leukaemic cells are highly susceptible to the action of antibodies *in vitro* and *in vivo*

Cells from spleen and lymph nodes are highly susceptible to the action of antibodies in the presence of guinea pig or rat complement *in vitro* Suspensions of bone marrow cells normally contain about 30 per cent of cells that are not damaged *in vitro*

Cells from thymus are highly susceptible to the cytotoxic effects of unheated guinea pig serum *in vitro* but less so to rat serum. Two leukaemias arising in the thymus showed a similar behaviour in their early transfers

The protective effect of homologous bone marrow and spleen in fatally irradiated mice may be abrogated by prior treatment with antibody It was shown that auto-antibodies and organ specific antibodies could not be implicated.

An attempt to use antibody production as an index of cellular survival for passive immunity experiments in intact animals was unsuccessful for technical reasons.

Splenic cells from one parent of an F_1 mouse immunised against the other parent may die out in F_1 hosts or proliferate and kill the host. The reason why parental splenic cells may sometimes die out in an F_1 is not yet determined.

If the inoculated cells do not die out, they rapidly invade the spleen and lymph nodes of the host and destroy the resident cells. The erythropoietic tissues are replaced more slowly

In all types of homologous disease the lymphoid cells of both donor and host are destroyed. It is suggested that death of the donor lymphoid cells is due to combination of host antigen with antibody attached to the donor cells (allergic death).

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DISCUSSION

OWEN. Is the cytotoxic antibody produced for example in C3H → A immunizations associated with an antigenic difference controlled by a factor in the H-2 complex.

GORER. Yes it is.

BRENT May I ask Dr Gorer whether he equates his cytotoxic antibody with the haemagglutinin the haemagglutinin seems to be a reasonable measure of the cytotoxin, though not the other way about. I wonder whether he really feels that he is dealing with one and same antibody

GORER That is a very difficult question to answer indeed All I can tell you is that if you do a genetic experiment typing say D and K using both tests, they will give you the same answer And that is far as I can go I do not quite know what else one can do

TRENTIN The observation that C57 lymphoid tissue, in contrast to A strain lymphoid tissue, does not persist when transplanted to the (A × C57) F_1 hybrid is very interesting It may explain my finding that strain A parental lymphoid tissue produces a significant degree of homologous disease in the unirradiated (C57 × A) F_1 weanling whereas C57 lymphoid tissue does not, even though protection of A-strain mice against lethal irradiation with C57 bone marrow results in severe homologous disease. I presume that the disappearance of C57 cells was not correlated with the sex linked antigen

GORER Those transfers were done by Boyse they were all between the same sex

VAN BEKKUM In your paper when you described the pathological effects of iso-immune A-strain cells injected into hybrids, there is a statement that lymph node cells are probably more rapidly lethal than splenic cells and furthermore that you saw no effect with thymus cells. Now we used a different experimental set-up we used irradiated mice which were treated with a curative number of homologous bone marrow cells and injected immediately afterwards with homologous lymph node cells, or spleen cells, or thymus cells. When a sufficient number of lymph node cells was injected the animals died within one or two weeks. So the beneficial action of the homologous bone marrow is prevented. The number of cells in the injected bone marrow was in itself sufficient to protect about 100 % of the mice this lethal effect is related to the number of lymphoid cells injected.

We have also found that the number of spleen cells needed to produce the effect is only slightly larger than the number of lymph node cells, but we need about 30 times more thymus cells to produce the same effect. This is in contrast to previous results we got with another experimental set up We injected rat bone marrow into irradiated mice and immediately afterwards isologous lymphoid cells this prevents

taking of the foreign graft, and in these experiments we have also titrated the number of lymphoid cells required we found that we needed only about twice as many thymus cells as lymph node cells to produce this effect. I have no explanation for this difference. Maybe the heterologous antigenic stimulus is greater in the latter case

GORER Well I think I might have an explanation for our failure and it is this that we have rather carelessly used a mixture of lymph node cells some of which have been draining ones and some of which were not and we may have had a lot of super-immunised lymph node cells, and very feebly immunised thymus ones, and of course there may be this dosage effect I think there is another thing that one might add. I am rather suspicious of the small thymocyte on the grounds of its reactions to guinea pig serum rather than to anything else But we are dealing with labile organs and perhaps I should have said that even doing a cytotoxic test on bone marrow for example you usually will get a 70 % kill every now and then you will get a 100 % kill I imagine because there are more susceptible cells due to the functional state of the organ One is up against that sort of difficulty in this kind of work I think

STETSON The production of skin reactions by cytotoxic sera might occur only if there were susceptible target leucocytes in the skin test area Have you tried to induce a local non-specific inflammation for instance by an initial intradermal injection of saline and then to produce a visible skin reaction by injecting cytotoxic serum into the site ?

GORER No I have not and I think it would be a very interesting thing to do

BRENT Concerning the immunological competence of thymocytes I should like to mention that Billingham and I have shown that C57 thymus cells intravenously injected into newborn A strain mice will cause runt disease with 100 % mortality This is true for normal non sensitized thymus cells The cell doing the damage may be the thymocyte itself or a cell type present in the thymus in small numbers we do not know but I think the presumption is that it is the thymocyte itself

Homologous disease in unirradiated F_1 hybrid mice receiving parental lymphoid tissue

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Mice protected against lethal doses of whole body irradiation by means of homologous or heterologous bone marrow develop a secondary disease the mortality from which is most severe during the second month Mice protected with isologous marrow do not develop this secondary disease Considerable evidence has been accumulated from several laboratories indicating that this disease results from an immunological (histo-incompatibility) reaction of successfully grafted foreign lymphoid elements, or their bone marrow progenitors, against the tissues of the host who has been rendered « tolerant » by virtue of the irradiation protection procedure[1] Similar mortality has been reported in unirradiated animals rendered tolerant by means of homologous spleen cell transplantation into the fetus or neonatal animal[illegible] If such disease entities are indeed caused by reaction of the grafted foreign lymphoid elements against the tissues of a tolerant host, similar mortality should theoretically occur also in unirradiated adult F_1 hybrids grafted with lymphoid tissues from the inbred parental strains, since the F_1 hybrid is « genetically tolerant » of tissue grafts from either inbred parental strain. Billingham, Brent and Medawar[3] have urged that possible reactions of a graft against its host should be kept in mind whenever normal tissues or tumors are transplanted from members of inbred parental lines into their hybrid progeny And indeed recently similar mortality has been obtained by transplantation of parental lymphoid tissue into unirradiated[illegible] and into sublethally irradiated[illegible] F_1 hybrid mice. Additional observations along this line of investigation will be presented

Unirradiated weanling (C57 × A) F_1 hybrid mice showed severe body weight loss and high mortality if injected intraperitoneally with combined spleen and lymph node cell suspension or lymph node cell suspension alone from the A parental strain (71 % and 68 % 60-day mortality res-

pectively) Spleen and lymph node from the C57 parental strain produced only 5 % mortality Spleen and lymph node from both parental strains combined produced 28 % mortality Isologous (F_1 hybrid) lymph node and spleen produced no mortality or weight loss. Whereas parental lymphoid tissue (A) produced high mortality in the F_1 hybrid spleen and lymph node from the (C57 × A) F_1 hybrid produced no weight loss or mortality in unirradiated A strain weanlings.

Bone marrow suspensions from the A parental strain or from both parental strains combined produced no mortality or weight loss in the unirradiated (C57 × A) F_1 hybrids.

Unirradiated weanling (A × Dba) F_1 hybrid mice showed severe body weight loss and high mortality if injected with lymph node and spleen from either the A strain (57 %) or from both parental strains combined (58 %) but only 8 % mortality with spleen and lymph node from the Dba parent Isologous (F_1) spleen and lymph node produced no mortality or weight loss.

Unirradiated weanling (BALB/c × CBA) F_1 hybrids showed weight loss and moderate mortality with spleen and lymph node from the CBA strain (33 %) or from both parental strains combined (15 %) but no mortality with spleen and lymph node from the BALB/c strain Isologous (F_1) spleen and lymph node produced no weight loss or mortality

Spleen and lymph node from the A strain which produced high mortality in the unirradiated (C57 × A) and (A × Dba) F_1 hybrids of the A strain produced no mortality in the (BALB/c × CBA) F_1 hybrid which does not have the A strain as a parent Similarly spleen and lymph node from the C57 and A strains combined produced mortality in the (A × Dba) and (C57 × A) F_1 hybrids which have one or both of the donor strains as a parent but produced no mortality in the (BALB/c × CBA) F_1 hybrid which has neither donor strain as a parent

Older F_1 hybrids (3 to 9 months of age) did not show the body weight depressing and lethal effects of a single injection of parental lymphoid tissue unless they were given a sublethal dose of irradiation prior to receiving parental lymphoid tissue However parabiosis of unirradiated older (C57 × A) F_1 hybrids with the A strain parent resulted in selective mortality of the F_1 hybrid Similar selective mortality of the F_1 hybrid parabiont has been reported by van Bekkum [1]

Severe tissue damage atrophy and fibrosis of lymphoid organs was observed in F_1 hybrids moribund as a result of parental lymphoid tissue injection

The term « homologous disease » was used by Barnes et al [1] to designate the delayed mortality observed in irradiated mice protected with homologous bone marrow or spleen without reference to the etiology of such disease Billingham has used the term « runt disease » to refer

to the disease and mortality seen following the injection of homologous spleen into fetal or neonatal animals [3, 7] The present experiments demonstrate that a comparable disease can be obtained by transplantation of parental lymphoid tissue into « genetically tolerant » unirradiated young adult F_1 hybrid mice or by parabiosis of adult parent and F_1 hybrid mice. A considerable amount of experimental evidence now supports the concept that all four of these disease entities result from a graft versus-host reaction [8], and a common terminology appears desirable It would seem appropriate to use the terms « homologous disease » and « heterologous disease » to designate the specific morbid processes resulting from a graft versus-host reaction attending the successful transplantation of homologous or heterologous as opposed to isologous or autologous, lymphoid elements or their progenitors into a tolerant host regardless of the reason for tolerance on the part of the host. A similar process should theoretically be observed following homologous lymphoid tissue transplantation into agammaglobulinemic hosts or hosts rendered immunologically unresponsive by any cause that would not similarly affect the transplanted homologous lymphoid tissue. Indeed it has recently been reported that repeated administration of large doses of homologous spleen cells into adult mice of an unrelated strain may in itself produce prolonged survival of skin homografts from the spleen donor strain atrophy of the lymphoid tissues body weight loss, and high mortality [4]

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DISCUSSION

ZAALBERG We have done nearly the same experiments with our two strains of mice the CBA and C57BL, and I should like to give you some of our results. Ten F_1 male hybrids (CBA♂ × C57BL♀) were grafted with parental male skin. About one month later these animals were irradiated with 400 r (a dose insufficient to kill these animals) and thereafter injected intraperitoneally with C57BL male spleen cells (10 per mouse). Twenty days later three animals sloughed the CBA skin whereas the C57BL skin remained normal Six animals including the three mentioned above, died within 41 days after injection of the spleen cells, with signs of emaciation and diarrhea. Rejection of the skin grafts shows that the injected spleen cells were able, by a graft-versus-host reaction to kill sublethally irradiated animals.

HAŠEK Dr Trentin in your parabionts between genetically compatible and non-compatible partners, did you investigate the weight of the spleen and what was the histological picture of the spleen of the partner which died?

TRENTIN In the parent to-F_1 hybrid parabiotic mice, we are presently collecting this information but it is too early to answer your question. In the unirradiated F_1 mice which were moribund as a result of a single transplant of parental spleen and lymph node there was lymphoid atrophy with loss of the normal lymphoid follicular organization of the spleen and subcutaneous lymph nodes. In some spleens, there were extensive degenerative changes.

PREHN Dr Trentin do you have any evidence or any idea of the amounts of vascular anastomosis in your parabionts?

TRENTIN I have not in any way attempted to measure it by dye transfer or marked red-cell transfer if that is what you have in mind We do inspect them at autopsy and find grossly good union The results also indicate that anastomosis was adequate to produce homologous disease.

PREHN I had in mind the fact that if it was adequate enough well then perhaps neither animal should die

OWEN I would like to speak briefly to that question on the basis of some work on parabiosis in rats which I did some years ago and which was mentioned in the Tolerance Conference published in the *Proceedings of the Royal Society* for 1956. This was a parabiosis between two kinds of rats that differed for a red-cell antigenic marker and in

which there did develop a tissue incompatibility leading to the autodisjunction of the members of the parabiotic pair But prior to this tissue reaction the parabionts underwent a free exchange and came into equilibrium with respect to their two cell types. Incidentally the red cells exchanged enjoyed a normal life span even after this disjunction which would suggest that they had not evoked an immune response for the red cell antigens with which they were marked

STETSON Have you tried to increase the resistance of weanling F_1 hybrids by transfusing them with adult isologous serum or lymphoid tissue before injecting the parental marrow ?

TRENTIN No I have not done that.

VAN BEKKUM With regard to the parabiosis experiments I want to point out a difference between the results Dr Trentin has just presented and the ones that we reported at the International Congress of Radiation Research in Burlington last year The majority of our CBA F_1 hybrid and C57BL-F_1 hybrid parabionts lived considerably longer than Dr Trentin's parabionts. We have omitted the animals that died before the 14th post-operative day from our data because we thought the mortality during that period to be mainly due to operation trauma technical failures, infection etc. There was also no obvious difference between the number of hybrids and of parental strain mice dying during the first two weeks However among the parabionts that survived the 14th day many lived for weeks or months and 3 out of 31 twins survived the 70th day post-operation. The distribution of mortality was as follows

Parabiont Combination.	Deaths between day 14 and 90		
	parental.	*F_1*	*both.*
CBA-F (17)	0	11	3
C57BL-F (14)	1	8	5

In a number of the F_1 hybrid partners of these twins surviving the 14th day skin lesions as well as wasting developed before death. These skin lesions were markedly similar to those observed in mice transplanted with foreign bone marrow I wonder whether Dr Trentin has observed these lesions in view of the short survival time of his animals.

TRENTIN As you saw from the slide, the particular combination studied did not live nearly this long In thirteen of the 14 pairs, the F_1 hybrid died and was separated from the parent. The average survival of the F_1 hybrid was only thirteen days. This was undoubtedly

related to the fact that this was selected as a very severe strain combination on the basis of results obtained with a single injection of lymphoid tissue. The much longer survivals you have obtained Dr Van Bekkum would seem to indicate a lesser degree of incompatibility giving a prolonged survival with time therefore for the chronic skin lesions to develop We did not see any comparable skin lesions With respect to the early mortality there is of course early mortality from surgical trauma in parabiotic experiments and we found that these had to be excluded. We found that a cut-off date of approximately 6 days post-operatively would distinguish non-specific deaths from those of a type which we feel result from an immunological reaction between the parent and the F_1 hybrid Our data excluded pairs dying within 6 days of parabiosis.

BRENT There is just one question Dr Trentin. In newborn mice the mortality of runt disease can be completely prevented by the use of bone marrow instead of spleen cells. Admittedly this does not mean at all that the symptoms of runt disease are suppressed because such mice although they do not die and grow moderately well may show involution of their lymphoid tissue to a greater or lesser extent I wonder why it is that homologous bone marrow injected into irradiated animals should be responsible for such a high mortality Might it be that by irradiating the animal a great deal of space is made available for the injected cells so that those bone marrow cells which are capable of immunological reactivity have an opportunity to proliferate and therefore cause greater injury to the host ?

TRENTIN I think some such explanation as this may be the basis for the difference you observe between bone marrow and spleen in the foetal tolerance experiments In the irradiation situation there is as you indicate undoubtedly a greater stimulus for regeneration and repopulation of lymphoid organs from the bone marrow graft a situation which probably does not exist in the unirradiated animal This presumably is the explanation for the difference which I observed in the unirradiated weanling F hybrids given either spleen or bone marrow from the parental strain Even in the A into (C57 × A) hybrid combination where the lymphoid tissue graft produced high mortality the bone marrow injection did not Strain A marrow used to protect lethally unirradiated C57 mice produces severe homologous disease

KLEIN On one of your slides Dr Trentin it seemed as if a mixture of isologous and homologous marrow would give better protection than isologous marrow alone I wonder if this is (1) a repeatable finding (2) if so what are the quantitative relationships between the two marrow components and (3) how do you interpret this

TRENTIN I failed to comment on this and I am glad you have given me the opportunity to do so The extent of difference of initial protection provided by the isologous marrow as compared to the isologous combined with the homologous was not great although the latter did give better protection. The simultaneous administration of isologous marrow with homologous bone marrow of a type that would produce homologous disease if given alone, did prevent the secondary occurrence of homologous disease. In this particular situation the proportions were roughly 50/50 that is equal amounts of isologous and homologous marrow I think that the absence of homologous disease is related to a prevention of repopulation with homologous elements as indicated by the fact that these mice subsequently rejected skin grafts from the homologous donor

KLEIN Does this happen if you work with unirradiated weanlings rather than with irradiated mice ?

TRENTIN This was done with irradiated animals. I have not done the combination that would be pertinent with the unirradiated weanling

BRENT Billingham and I have come across an interesting example of cell selection which I think is relevant to some of the previous comments. C57 spleen cells injected into A line newborn mice are always lethal But when a mixture of equal numbers of CBA and C57 cells is injected intravenously into newborn A line mice the presence of CBA cells in the inoculum protects the A line animals from the graft versus host reaction that would normally be directed against them by the C57 cells. If these animals are later tested for tolerance they are found to be tolerant of CBA skin grafts, but not of C57 Here a graft versus graft reaction has eliminated the C57 cells the CBA cells having been at a selective advantage because they find it easier to establish tolerance —a fact which we know from other experiments.

LOUTIT I doubt whether we need invoke in every instance the immunological reaction to the exclusion of all others. For instance our experiments are with the transfer of marrow to the lethally irradiated mouse Even though it contains a number of lymphoid cells, I do not think that those lymphocytes directly repopulate the lymphoid system of the irradiated host As Trentin has observed marrow plus spleen from an immunized animal gives an accelerated homologous disease, but plain marrow only from an immunized animal does not give any acceleration

The graft versus host assay in transplantation chimaeras

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Introduction

Every homotransplantation is in fact a chimaera formation: the shaping of a new individual composed of cells of different genotypes.

The transplantation chimaeras with which we are concerned in the present communication, however, have been produced by transplantation of reticulo-endothelial and haematopoietic cells to recipients which are for some reason or other immunologically incompetent at the time of transplantation. In this case the transplanted cells are not rejected by the usual immune reaction of the host and are therefore allowed to display their own immunological capabilities, whatever these might be.

It was expected from theoretical considerations, and amply verified by experiment, that *adult* r-e cells, such as spleen and lymph node cells, *will be stimulated by antigens of the host to react against the latter in a truly immune or hypersensitive way.* *This effect requires that the* genetic situation is such that the host contains antigens which are absent in the donor (contrarywise, the presence of donor antigens which are absent in the host does not under these circumstances lead to any *reaction*). *If on the other hand the transplanted cells are young enough* at the time of transplantation they would be expected to become tolerant of their host instead of immune. Hence the morphological and other reactions which are characteristic for the graft vs. host reaction of similar adult cells, should be absent.

The biological situation which arises when a r-e chimaera is formed is likely to be much more complicated than that appearing from the

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sketchy outline above. We consider it important therefore, to be able to make use of a simple and standardized procedure of graft vs. host reaction in infant recipients which in many ways should facilitate the study of what is really happening in transplantation chimaeras. The procedure has earlier been described as the graft vs. host assay of histocompatibility and the present paper is meant to show the following facets of the assay

(1) Reproducibility and time relations within a single genetic system.
(2) A gene dose effect in the graft vs. host reaction
(3) Analysis of antigenic composition of chimaeric tissues
(4) Use of the graft vs. host assay in design of irradiation chimaeras.
(5) Analysis of immunological reactivity of chimaeric tissues

(1) Reproducibility and Time Relations within a Single Genetic System

The theory and procedure of the graft vs. host assay has been described recently[9, 14] Essentially it consists in recording the spleen and liver enlargement which results in suitable recipients from transplantation of adult spleen cells. In the original version of this test[13, 14] it was necessary to do the grafting before or within the first day after birth (mice and chicks) because the power of the host defence against the graft starts to develop so early that a few day s delay after birth will preclude any noteworthy graft vs. host reaction.

The use of recipients which are first generation hybrids between the donor strain and the other strain against which the donor is expected to react, overcomes this practical inconvenience. At the same time it assures that if any effect is observed, it will be due to dominantly determined antigens[14]

Some technical details The cellular suspension is made in a small siliconized glass vessel by first cutting the spleen with scissors into small fragments. During this process a drop of Tyrode s solution is added to prevent the tissue sticking too firmly to the instruments.

Next, another ½ 1 cc. of Tyrode s solution is added and further dissociation of cells is obtained by taking up the fragments in a syringe without needle and pressing the contents out against the bottom of the glass. This is repeated a few times increasing the firmness with which the tip of the syringe is held against the glass. The resulting suspension is filtered through a tiny piece of gauze which effectively retains anything which cannot with ease be injected with a 20-gauge needle. A count of nucleated cells is made after staining with methyl violet in acetic acid and the suspension is adjusted to contain the desired number of cells in 0.1 cc.

The intraperitoneal injection of even that small volume may in n born mice lead some of the fluid to escape again unless some spe precaution is taken. A safe and handy way is to insert the tip of needle through the right m. triceps and to push it subcutaneously it passes below the lower edge of the liver Only then is the abdom wall pierced. After injection the needle should be withdrawn gently as not to widen the peritoneal opening If nevertheless some fl escapes into the subcutaneous channel produced by the needle, it is ciently blocked by the « water-lock » formed by the leg muscle Dur this handling the little mouse is conveniently laid on its back and fi by the operators left hand taking the head between the 1st and fingertips and stretching the body and right hind leg with the finger any assistance being preferably avoided.

Controls			
mg/100g:	295	330	323
Injected			
mg/100g:	1410	1240	1440
Index 1	4 50	3 96	4 60

FIG. 1.

The experiments in the present section were all performed with t same immunogenetic system adult C_3H spleen cells injected i-p in 1 11 day test litters of F_1 hybrids between C_3H and DAB/2 This co bination is one of strong dominantly determined differences Sple and liver indices were recorded as earlier described [14]

Fig 1 (which shows one of the litters entered in table II) serves illustrate the gross appearance of the spleens and their correspondi weights and indices in a typical graft vs. host assay

The variables investigated in this section are (a) the age of te litters at the time of grafting (b) the length of the period betwe grafting and sacrifice and (c) the amount of tran planted cells

Significance of age of test litters at the time of grafting

Table I shows the means and variances of spleen and liver indic in litters which ranged from 1 to 11 days of age when grafted Fiv litters were 1 4 days, and five others were 7 11 days old.

It is seen that the younger of these groups showed consistentl higher liver indices than the older one while there is con iderable ove

lapping in the corresponding spleen figures The t test applied to the 2 groups proves the difference to be significant for livers at the 0.1 % level but quite insignificant for spleens. In each group there is a tendency for intra litter variance to be less than the variance between litters. This is especially pronounced for spleens and is a tendency which is also seen in tables II and III

TABLE I

Spleen- and liver indices at 10 days after injection of 10^7 nucleated adult C_3H spleen cells into $(C_3H \times DBA)$ F_1-hybrids

Age of litter at injection Days	Number of injected animals	Spleen		Liver	
		Mean index.	Variance	Mean index.	Variance.
1	2	2.99	0.0162	1.75	0.0050
3	2	3.10	0.0420	1.95	0.0221
4	3	3.64	0.0003	1.58	0.0201
4	2	4.06	0.1152	2.02	0.0112
4	4	3.37	0.0581	1.84	0.0090
Inter litter means and variances		3.43	0.2208	1.82	0.0324
7	3	4.39	1.1023	1.43	0.0046
8	4	2.31	0.2020	1.25	0.0521
8	3	2.06	0.1056	1.27	0.0248
9	3	2.28	0.0252	1.24	0.0018
11	2	2.78	0.0648	1.10	0.0150
Inter litter means and variances		2.96	0.7500	1.26	0.0128

TABLE II.

Spleen and liver indices at 8 days after injection of 10^7 nucleated adult C_3H spleen cells into $(C_3H \times DBA)$ F_1-hybrids

Age of litter at injection Days	Number of injected animals	Spleen		Liver	
		Mean index.	Variance.	Mean index.	Variance
4	2	3.19	0.0098	1.85	0.0025
4	3	3.09	0.0418	1.78	0.0076
4	2	3.68	0.0032	1.80	0.0008
5	3	3.57	0.0150	1.62	0.0250
6	2	4.25	0.0800	1.55	0.0060
6	2	3.10	0.0032	1.42	0.0018
7	3	2.47	0.0095	1.19	0.0008
8	3	4.35	0.1185	1.29	0.0004
Inter litter means and variances		3.48	[illegible]	[illegible]	

The variance is calculated by the formula

$$\frac{s(x^2) - \frac{(sx)^2}{n}}{n - 1}$$

where n is the number of observations, sx and s (x^2) are respectively the sum and the sum of squares of observations.

These investigations have not yet been extended systematically into the age range of 10-30 days. When however the same amount of cells were injected i.v. into hybrid adults there was no enlargement of the

TABLE III

Spleen and liver indices at 6 days after injection of 10^7 nucleated adult C_3H spleen cells into $(C_3H \times DBA)\ F_1$-hybrids

Age of litter at infection Days.	Number of infected animals.	Spleen		Liver	
		Mean index.	Variance	Mean index	Variance
1	6	1.54	0.0070	1.30	0.0045
1	8	1.28	0.0265	1.29	0.0031
1	4	1.45	0.0079	1.48	0.0001
1	2	1.63	0.2592	1.25	0.0113
8	3	1.92	0.0058	1.24	0.0030
11	3	2.30	0.0076	1.36	0.0020
Inter-litter means and variances	–	1.70	0.1283	1.30	0.0080

liver after a 10 day period and spleen enlargement was very capricious. In 2 out of 7 litters there was a mean spleen index of 1.74 and 1.57 respectively. In the remaining 5 litters there was no enlargement whatever. It could admittedly be argued that a higher dose of spleen cells might equally have produced splenomegaly in adults. Nevertheless on a pure dose to body weight basis there is no doubt that 10^7 cells would be sufficient to produce a constant spleen enlargement if there were no other factors involved. We must admit that we do not understand why this phenomenon is not as reproducible in adults as in infant hybrids.

A possible explanation so far without support would be that recessively determined transplantation antigens in the grafted spleen cells elicit a host vs. graft reaction which precludes the graft vs. host reaction. This hypothesis is not only in conflict with available evidence from skin grafting, but would also imply that recessively determined antigens are developed later in life than the dominantly determined ones.

Significance of the period between grafting and sacrifice

Fig 2 shows for 4 infant litters of 8 animals each the development of spleen and liver indices with time after injection In each litter 4 animals were injected i-p with 10^7 spleen cells and four were kept as normal controls.

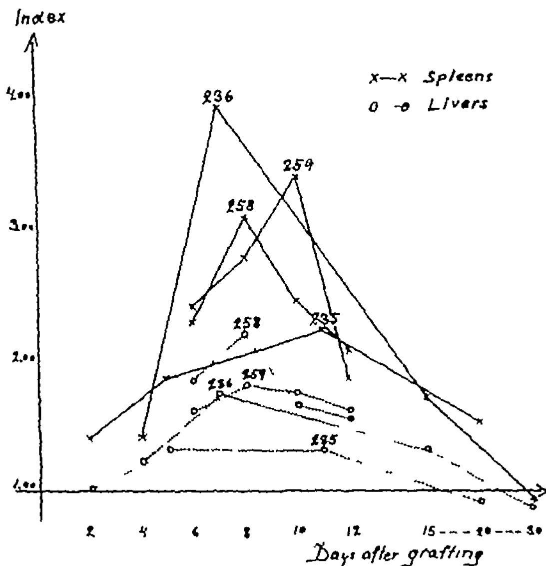


FIG 2

In litters 258 and 259 (2 3 days old at the time of injection) one injected and one control were sacrificed 6, 8 10 and 12 days later In litters 325 and 236 (7-8 days old at the time of injection) larger intervals were chosen within the range of 20-30 days. The results leave no doubt that spleen and liver indices alike have a peak at some 7 10 days after injection. It is not surprising that the maximum reaction should be reached by this time. It is a source of more surprise that the reaction fades away again as it seems to do after some 20-30 days

This is in fact another point which is not properly understood. A very likely possibility is that the reacting graft cells are destroyed in consequence of their own reaction with host antigens which must be abundantly available in their immediate vicinity The main point of interest here is whether immunologically competent graft cells persist at all after a month's time This is a crucial point which has not yet been investigated but the question can probably be settled by applying the principles outlined in section 5 The possibility cannot be excluded that even adult spleen contains some cells which are sufficiently immature *to acquire tolerance. Naturally* such cells would never reveal their presence in a usual immunological procedure, because the net result would then be determined by those members of the population which are capable of an immune reaction On the other hand during the later *stage of a graft vs. host reaction there might conceivably be a selective* immunological pressure in favour of cells which are not sufficiently mature to become immune. This is in fact a problem of great biological importance it amounts to the question whether or not r-c cells during their individual life cycle in adult individuals recapitulate the ontogenetic phases of immunological behaviour

In Tables II and III results are given from other litters which were sacrificed in toto at 6 or 8 days after injection with 10⁷ cells. Comparing the Tables I III it is obvious that the spleen index is higher at the 8th and 10th day than at the 6th day The difference between spleen indices at the 8th and 10th day is, however quite insignificant A similar trend can be seen for the liver figures when account is taken of the age at grafting (which as mentioned is of more importance for the liver index).

Significance of the number of grafted cells

A comparison of Tables I and IV shows that for litters injected at 1 4 days of age - mill cells usually produce lower indices than 10 mill cells. The t test applied to the 2 groups of data shows the differences to be significant at the 0 1 % level for both spleen and livers In spite of statistical significance however the difference does not seem sufficiently marked to allow a titration of reacting cells within the range investigated.

The minimum amount of cells required to elicit increased indices has not been determined for spleen cells. With lymph node cells (cf section 3) the minimum necessary is of the order of 10⁵

In concluding this section the following points should be made the grafts vs host assay is a very reproducible test of transplantation reaction when certain time limits are observed notably that hybrid litters are not too old (preferably less than 10 days) and that it sacrificed sufficiently close to the peak of the reaction (after [6]

[11] days) All experience we have gathered so far from immuno-genetic systems, other than that dealt with in section 1 seems to fit these requi rements

TABLE IV

Spleen and liver indices at 10 days after injection of 2×10^6 nucleated adult C_3H spleen cells into $(C_3H \times DBA) F_1$-hybrids

Age of litter at infection Days	Number of injected animals	Spleen		Liver	
		Mean index.	Variance	Mean index.	Variance
1	2	2.84	0.8628	1.52	0.0040
1	3	2.68	0.1626	1.41	0.0678
1	2	2.28	0.0392	1.32	0.0005
2	2	2.81	0.0006	1.56	0.0098
3	3	2.03	0.0180	1.22	0.0006
4	3	2.17	0.3441	1.46	0.0122
4	3	2.65	0.3707	1.38	0.0187
Inter-litter means and variances		2.64	0.1434	1.42	0.0161

The graft vs host assay is essentially an utilization for analytical purposes of two early signs of the systemic disease for which Billingham and Brent coined the term « runt disease » It is important to note, however that « runting » in the sense of general wasting is a far from constant feature in the animals used in the present studies. In fact, the body weight of injected animals rarely departs significantly from that of their normal litter mates. This is advantageous for statistical reasons because the method of calculating indices implies that loss in body weight introduces an error which it is difficult to eliminate.

The graft vs host assay is not simply a quicker method than waiting for « runting » and possible death to occur It is also presumably less dependent on irrelevant factors such as complicating infections it is more readily quantitated and undoubtedly far more sensitive.

(2) *A Gene Dose Effect in the Graft vs Host Reaction*

In our paper presented at the Third Tissue Homotransplantation Conference in New York [11] an addition was made in galley proofs stating that evidence was being accumulated of recessively determined antigens in C3H mice which could elicit a graft vs host reaction in injected AKR-cells. Thus the development of secondary disease in irradiated C3H mice treated with adult AKR spleen cells was ascribed to such antigens, which would be expected to escape detection in the graft vs host

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In Tables II and III results are given from other litters which were sacrificed in toto at 6 or 8 days after injection with 10^7 cells. Comparing the Tables I-III it is obvious that the spleen index is higher at the 8th and 10th day than at the 6th day. The difference between spleen indices at the 8th and 10th day is, however, quite insignificant. A similar trend can be seen for the liver figures when account is taken of the age at grafting (which, as mentioned, is of more importance for the liver index).

Significance of the number of grafted cells

A comparison of Tables I and IV shows that for litters injected at 14 days of age 2 mill. cells usually produce lower indices than 10 mill. cells. The t test applied to the 2 groups of data shows the differences to be significant at the 0.1 % level for both spleen and livers. In spite of statistical significance, however, the difference does not seem sufficiently marked to allow a titration of reacting cells within the range investigated.

The minimum amount of cells required to elicit increased indices has not been determined for spleen cells. With lymph node cells (cf. section 3) the minimum necessary is of the order of 10^5.

In concluding this section the following points should be made: the grafts vs. host assay is a very reproducible test of transplantation reaction when certain time limits are observed, notably that hybrid test litters are not too old (preferably less than 10 days) and that they are sacrificed sufficiently close to the peak of the reaction (after [6] 7 to 10

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TABLE IV

Spleen and liver indices at 10 days after injection of 2 × 10⁸ nucleated adult C_3H spleen cells into $(C_3H \times DBA) F_1$-hybrids.

Age of litter at injection Days	Number of injected animals.	Spleen		Liver	
		Mean index.	Variance	Mean index.	Variance
1	2	2.84	0.3528	1.62	0.0040
1	2	2.68	0.1625	1.41	0.0578
1	2	2.26	0.0392	1.32	0.0006
3	2	2.83	0.0006	1.58	0.0098
3	2	2.03	0.0180	1.32	0.0006
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(11) days) All experience we have gathered so far from immuno-genetic systems, other than that dealt with in section 1 seems to fit these requirements

TABLE IV

Spleen and liver indices at 10 days after injection of 2 × 10⁶ nucleated adult C_3H spleen cells into $(C_3H \times DBA)F_1$ hybrids

Age of litter at infection Days	Number of injected animals	Spleen		Liver	
		Mean index	Variance	Mean index	Variance
1	2	2.84	0.3628	1.62	0.0040
1	2	2.68	0.1825	1.41	0.0678
1	2	2.28	0.0392	1.32	0 0006
3	2	2.83	0.0006	1.58	0.0098
3	2	2.05	0.0180	1.22	0.0005
4	3	3.17	0.2441	1 48	0.0122
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Inter litter means and variances		2.64	0.1434	1 42	0.0151

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assay previously performed with grafting to hybrid recipients. Our present evidence is such that we shall withdraw the term « recessively determined antigens » in this context The facts are better described as a case of gene dose effect in a dominant but weak histocompatibility system.

The gene dose effect shows up as a difference in spleen and liver indices between (C3H × AKR) F_1 hybrids and C3H mice injected within 24 hours of birth with 10 mill. nucleated spleen cells from adult AKR donors and sacrificed 11 days later (Table V).

TABLE V

Group	I		II		III	
	Donor AKR	Host C H	Donor AKR	Host (C H × AKR)	Donor (C H × AKR)	Host C H
C H-Genes	O	+ +	O	+	+	+ +
	Spleen	*Liver*	*Spleen*	*Liver*	*Spleen*	*Liver*
Indices	1.75	1.31	1.07	0.98	1.10	0.97
	1.75	1.22	1.11	1.07	0.86	0.92
	1.99	1.27	1.20	0.91	1.42*	1.01
	1.44	1.13	1.53	0.96*	0.99	0.97
	1.90	1.54	1.02	0.92	1.31	0.96
	2.28	1.34	1.19	0.90	0.92	0.96
Mean	1.85	1.32	1.17	0.96	1.08	0.97
Standard Error	0.28	0.14	0.17	0.06	0.21	0.04
$\frac{\text{S.E.} \times 100}{\text{Mean}}$	15.1	10.6	14.6	6.1	19.4	4.1

( ) From litters where only one animal was injected. The fact that the moderately increased spleen is not accompanied by enlarged liver detracts from the value of these particular indices

Each index entered in Table V is the mean of the indices obtained from a whole litter which as usual consists of an approximately equal number of injected and control animals The t-test applied to the data in groups I and II shows that spleen and liver indices alike are significantly higher in the former at the 0.1 % level It can be argued that a litter of 3 injected animals should be given more weight in statistical analysis than a litter of 1 or 2. Actually the intra litter differences are such that it makes the group differences even more significant if the t test is applied to the entire samples of indices.

The test for detection of recessively determined antigens which was earlier suggested ([1] fig 1) consists in transplantation of adult F_1 hybrid spleen (in casu C3H × AKR) to newborn recipients of parental strain origin (in casu C3H). As seen from Table V group III there is with this method no proof of a graft vs. host reaction However there are in theory grounds to suspect that the method could fail even if recessively determined antigens were in fact present It could thus be postulated that the presence of a single gene dose of C3H origin in the hybrid donor would function as a self marker in the sense of Burnet's and Fenner's concept[8] Hence the graft would be naturally tolerant of the host in spite of the recessive antigen being present in the host and absent in the donor Therefore though the date of Table V could *per se* still be interpreted in terms of recessively determined antigens, a crucial test to discriminate between this possibility and that of a gene dose effect in a weak dominant system would be to pre-immunize the AKR donor with hybrid cells. Schematically the test is designed as follows

1 *Stage* Adult AKR, three i-p injections, at two week intervals of spleen cells from normal adult (C3H × AKR) F_1

2 *Stage* Adult AKR (immunized) and AKR (normal) donors Transplantation of 1 mill spleen cells from each to different members of the same (C3H × AKR) F_1 litters.

Data from such an experiment involving 5 infant recipient litters are shown in Table VI The litters were sacrificed two days after injection

TABLE VI

(*Each index represents the mean value for one litter*)

Age of litter at grafting (Days)	*Group I* Donor Immune AKR	Host (C H × AKR)F	*Group II* Donor Normal AKR	Host (C H × AKR)F_1
	Indices Spleen	Liver	*Indices* Spleen	Liver
8	1.70	1.35	1.07	1.00
7	1.91	1.24	0.95	1.00
1	1.77	1.44	1.10	1.00
6	2.58	1.29	1.00	1.08
7	2.23	1.26	1.06	1.00
Mean	2.04	1.32	1.04	1.02
Standard Error	0.36	0.09	0.05	0.05

It is clear that a marked graft vs. host reaction followed the transplantation of AKR-cells which were pre-immunized with hybrid cells

but not the grafting of normal ones Therefore transplantation antigens of C3H origin must be present in the hybrid spleen. Hence the *antigens are dominantly determined in the sense of* expressing the effect of a single gene dose. Their relative weakness is revealed by the fact that they need a sort of « secondary response » to manifest themselves

The demonstration in transplantation immunity of a gene dose effect in a dominant system is of no less interest than our earlier claim to have met an antigen governed by purely recessive genes. There is no reason to anticipate that the gene dose phenomenon is restricted to weak antigens but we have not yet extended to other systems a systematic search for a similar effect. *However there is probably an indication* of this kind in the fact that we have repeatedly observed a patchy macroscopic liver necrosis in homozygous mice which were injected as newborns with spleen cells from an incompatible strain but not in newborn hybrids injected in the same way In either case spleen and liver indices were of course largely increased

(3) *Analysis of Antigenic Composition of Chimaeric Tissues*

The demonstration in the preceding paragraph that the graft vs. host reaction can be speeded up by previous contact of donor cells with the host antigen has some further implications.

Firstly it is one more decisive piece of evidence that the spleen and liver enlargements are actually caused by an immunological attack on the host by the grafted cells and not vice versa

Secondly it offers in theory a good possibility to develop a new method for analyzing presumed chimaeric tissues with respect to the transplantation antigens of which they may be composed

Let us assume that we want to check whether donor antigens (D) are still present in a mouse (R) which has earlier been grafted from a D-mouse. In principle this should be possible by immunizing an adult D-mouse with the presumed chimaeric tissue and subsequently grafting the immunized spleen or lymph nodes to infant (D × R) F_1 hybrids, under conditions such that a normal D donor would not cause a spleen or liver enlargement In practice this also seems to be pos ible in genetic systems with strong antigenic differences

For this purpose regional lymph node cells have been grafted instead of spleen cells because the amount of chimaeric tissue needed for sensitizing a regional lymph node was presumed to be less than for sensitizing the spleen.

Preliminary ex[illegible] ts with norm[illegible] ph nodes injected i p into infant (ST [illegible] F_1 test litter [illegible] hat 10 cells gave no reaction 3 × 1 [illegible] a spleen en[illegible] on the borderline of

significance (indices around 1.30) without liver enlargement and 10^6 cells gave significant increases of both spleen and liver indices. All litters were sacrificed after 6 days.

A dose of 10^5–3×10^5 cells was therefore considered appropriate for the next step: grafting of immune lymph nodes. Five adult ST males were injected s-c in the feet with various doses of spleen cells from a normal DBA/2 male. After 4 days regional lymph nodes were removed and made into cellular suspensions. Lymph nodes from a normal ST male were included as control. These 6 cell preparations were then injected i.p. into 3 infant litters of (ST × DBA/2) F^1 hybrids of 6 members each. In each litter there was thus one mouse per donor. The number of injected cells in one litter was 10^5 and in two others was 3×10^5. The litters were sacrificed 7 days later and spleen and liver indices were calculated taking the litter-mate injected with normal cells as control in each litter.

The results are entered in Table VII.

TABLE VII — *Antigen analysis by means of pre-immunized donors*

Number of ST lymph node cells injected in test-litters	*Number of DBA/2 spleen cells injected in ST donor*									
	10^4		10^5		3×10^5		10^6		10^7	
	Spl. ind.	Liv. ind.	Spl. ind.	Liv. ind.	Spl. ind.	Liv. ind.	Spl. ind.	Liv. ind.	Spl. ind.	Liv. ind.
10^5	1.05	1.01	2.16	1.56	2.11	1.20	2.03	1.30	2.79	1.50
3 × 10	0.94	1.05	2.28	1.43	1.93	1.36	2.06	1.28	2.80	1.48
3 × 10	0.91	0.97	0.88	1.03	1.78	1.36	1.99	1.35	2.02	1.31

Having satisfied ourselves that the graft vs. host assay allowed us to discriminate between normal and immune cells, under the conditions defined, we next tried this procedure with a single irradiation chimaera (328/4, which is mentioned in section 5). Two prospective ST donors were injected with spleen cells from the chimaera, and regional lymph nodes subsequently grafted to appropriate test litters (ST × C_3H) F_1 hybrids. The results clearly indicated that chimaera must have contained C_3H antigens but this was not surprising in fact since we already knew from another assay described in section 5 that living C_3H cells were present in the chimaera.

The procedure described here may possibly be useful in testing for the presence of donor *antigens* in supposed chimaeras when other methods have failed to reveal the presence of donor *cells*. The test, when

positive is probably not a proof of living cells, which are better demonstrated by other means.

(4) Use of the Graft vs Host Assay in Design of Irradiation Chimaeras

It is firmly established by several independent methods that the curative effect on otherwise lethal irradiation disease from transplantation of bone marrow or spleen cells is due to recolonization of lymphoid and r-e tissues by the grafted cells[2, 7, 8, 11, 12]

There is abundant proof today that the therapeutic effect is highly dependent on the genetic relationship between donor and irradiated host. Thus, when recipient and donor belong to the same inbred strain the protection is much superior to that usually obtained by homotransplantation When bone marrow is the grafted tissue, this difference can be seen in the need for injection of a bigger amount of homologous cells to obtain the same survival rate as that given by a smaller amount of isologous cells[3]

*Most dramatic are the different long term results in homo- and iso-*transplantation of adult bone marrow when either have protected well for the first 3 dangerous weeks (the time of primary irradiation disease). While isologous cells can secure a fairly normal existence for a life span which is but little shortened, homologous cells lead to an usually fatal secondary disease generally coming on 1 2 months after the grafting[1, 8, 14]

It is very significant that the development of secondary disease seems to require that the host contains antigens which are absent in the donor and therefore can provoke the grafted cells to attack their host[1, 1] Again the reverse genetic situation (which would allow a host vs. graft reaction) is supposed to be of no importance provided that the irradiation dose has been high enough to abolish the recipient's immune response

We have previously published preliminary facts in support of the idea that the graft vs host assay gives a way to predict the outcome of r-e cell grafting to irradiated mice[1] Thus for the C3H strain tested in combination with 3 other strains, the assay pointed to considerably more compatibility with AKR than with ST or DBA. This finding appeared well supported by a small irradiation experiment in which C3H mice were exposed to an X ray dose of 85 % mortality in *the controls. When adult AKR spleen cells were injected* the mortality after 30 days was decreased to 35 % whereas adult ST spleen cells increased the mortality to 100 % and even shortened the death time.

While the 3 surviving control mice are still alive and healthy after 12 months all 13 survivors from the AKR treatment have later died

from secondary disease. It is our experience from this and later experiments with the same genetic system that secondary disease can develop at any time from 1 1/2 to 12 months after irradiation. In all probability the danger of developing this condition remains for the rest of an almost normal life span. It is very spectacular that the mice may appear entirely normal for many months including a normal greying of the pelt before symptoms like wasting and diarrhoea set in. This may seem strange on immunological grounds but hardly more so than the very delayed skin homograft rejection caused by weak histocompatibility differences.

Table VIII gives data from new and more extensive studies of the same nature, except that the irradiation dose is now in the supralethal range (1200 r).

Again it is found that adult AKR spleen gives at least some protection from primary disease whereas adult ST and DBA spleens, if anything, accelerate death.

When infant donors (about 1 week old) are used, similar differences according to strain relationship are found at a much higher level of beneficial effect. Now the ST and DBA donors protect well against primary disease, but death occurs in 1-2 months from secondary disease in all but one case (of which the donor exceptionally was as young as 2 days). AKR donors, at the other hand, afford long lasting recovery as a predominant result, though quite a few have succumbed in a 9-month period to a late secondary disease.

The use of foetal liver cells was expected to give a further improvement in protection, in the « incompatible » combinations also. For reasons which are not understood both ST and DBA cells failed to protect even against primary disease. There is no indication of faulty technique, since foetal livers gave perfect protection of at least 8 month's duration when derived from isologous C3H or from the relatively compatible AKR strain.

It is strange that foetal liver from ST and DBA gives less protection than infant spleen of the same extraction. From a « graft vs. host point of view » the opposite should rather be expected as it would from a pure « host vs. graft point of view ».

It was thought that a higher dose of foetal livers cells from the two incompatible strains might possibly change the picture. The lower horizontal column of Table VIII shows that it does to a certain extent. Firstly more early survivors are obtained. Secondly some of the early survivors are also alive at 3 1/2 months (time of writing). Some developed transitory signs of secondary disease while others were killed or died under this diagnosis.

TABLE VIII.

Grafting of spleen or foetal liver cells to C3H recipients after 1200 r of continuous 24 h whole body irradiation.

		Cells Mill.	No. Mice	Survivals at ½ month.	2 months.	3 months.	6 months.	9 months.	Predominant result.
Nil		0	81	0	0	0	0	0	Primary disease.
Adult Spleen	AKR	10	25	7	5	4	4	2	Primary or secondary disease
» »	ST	»	12	0	0	0	0	0	Primary disease.
» »	DBA	»	30	1	0	0	0	0	Primary disease.
Infant Spleen	C H	»	14	13	13	13	13	13	Recovery
» »	AKR	»	17	17	16	15	14	13	Recovery or secondary disease.
» »	ST	»	12	11	3	0	0	0	Primary disease delayed
» »	DBA	»	24	24	2	1	?	?	Primary disease delayed.
Foetal liver	C H	»	26	19	19	19	19	?	Recovery
» »	AKR	»	15	12	12	12	12	?	Recovery
»	ST	»	26	1	1	1	killed at 4 ½ months		Primary disease.
»	DBA	»	26	0	0	0	0	0	Primary disease.
Foetal liver repeated injections	ST	35	13	1	0	0	0	0	Primary disease.
» »	ST	70-77	13	6	4	?	?	?	Primary or secondary disease
» »	ST + DBA	98	10	4	3	?	?	?	Primary or secondary disease
» »	DBA	25-30	15	4	3	2	?	?	Primary or secondary disease.
Single injection	DBA	25-30	16	0	0	0	0	0	Primary disease

The development of secondary disease after primary protection with foetal homologous liver was earlier observed by Trentin (discussion of [13]) This finding can only be reconciled with a graft vs. host etiology if one of two postulates are made

(1) Certain antigenic differences in homologous systems are of such a kind that acquired tolerance cannot be achieved

2) These same antigenic differences require for induction of tolerance that exposure is made very early in embryonic life

Either postulate may well prove correct since it is documented already that tolerance of homologous skin is induced with great variation of success in different mouse strain combinations [4]

Successful protection with foetal homologous liver has been reported by several investigators [2, 16, 18] It would be important to know not only the long term results, but also the out-come of graft vs host assays performed with the employed strain combinations.

The best results obtained by these investigators are those of Uphoff [18] Her experimental conditions are unique however in the sense that irradiated F_1 hybrids were used throughout for foetal liver grafting from parental strains. It might be, therefore that she has in fact taken advantage of a gene dose effect which reduces the antigenic stimulus to the graft.

Our own experience of grafting with foetal livers is very discouraging, except in the combination of C_3H and AKR where it works beautifully either way (details of grafting of foetal C_3H liver to irradiated AKR will be given in a later communication) As mentioned earlier this is the only strain combination in our present stock of mice where the graft vs. host assay performed with hybrid litters reveals a relative compatibility

Of some 50 lethally irradiated ST mice, not a single one has been rescued from grafting of foetal cells whether from C_3H DBA/2 or AKR. The treatment has not even protected against primary disease. The very poor results from grafting irradiated C_3H mice with foetal ST or DBA/2 liver have already been mentioned (Table VIII)

In fact these results from incompatible strain combinations are rather suggestive of the possibility that the irradiation did not entirely abolish the immunological host defence (in spite of 100 % lethality in controls) To our knowledge, we are the only investigators who have routinely employed a 24 h continuous irradiation procedure in this type of experiment. All others seem to have used a much higher dose rate

The possibility should therefore be investigated that a lethal dose of low rate is less suppressive of transplantation immunity than a higher dose rate with the same effect on haematopoietic cells. We will try to

elucidate this question by performing graft vs. host assays in appropriate test litters with spleens from irradiated but otherwise untreated mice taking the dose rate as variable.

The curious fact mentioned above that infant spleens from incompatible donors in our hands gave better protection than the corresponding foetal cells, may conceivably have a rather sophisticated explanation which is based on an inter play between host vs. graft and graft vs. host forces. Thus, if immunological reactivity of the host is not entirely abolished by irradiation the grafting of incompatible infant spleens may provide that gentle extra blow to the r-e system of the host which would be sufficient to enable the graft to establish itself to protect through the primary phase and to kill by a graft vs. host reaction which is diagnosed as secondary disease

It can by now be added thanks to the kind help of Drs P.A. Gorer and B Mikulska that the H 2 groups of our mouse strains are H 2k for C_3H and AKR, H 2d for DBA/2 and H 2b for ST This latter finding was a surprise because what in USA is called ST has earlier been typed by Dr G Snell as H 2k

It thus appears that the grouping in compatible and incompatible pairs, which we had ourselves made on the basis of graft vs. host assays [15] entirely fits the H 2 grouping It does not follow of course that the two groupings will always be congruent, and it is very desirable that comparison should be made in further strain combinations.

(5) *Analysis of Immunological Reactivity of Chimaeric Tissues*

Like section 3 the present one deal with the incipient development of the graft vs. host assay for a new purpose. Let us again consider a mouse (R) which is suspected of or proved to be chimaeric from previous grafting with homologous r-e cells from a donor (D)

The problem is now to design an experiment which can discriminate between the immunological reactivity of R and D cells in chimaeric tissues, such as spleen and lymph nodes.

The information wanted is not only the capacity of the two kinds of cells to react against each other's antigens but also their reactive power in respect of a third strain (T) against which they would normally react

In theory this purpose is met by the following design (Table IX) which involves 4 different test litters per chimaera

In the newborn R litter only D-cells of the chimaeric tissue can possibly cause an enlargement of spleens and livers. The test requires, however kinds of appropriate controls viz (1) the usual non injected litter mate controls and (2) other litter mates injected with r-e cells from a normal D-mouse The latter kind of control is necessary

because experience has shown that not all homozygous litters are sufficiently immature to allow the grafted cells to display their graft *vs.* host reaction. A negative result from injection of chimaera cells is hence significant only of D-cells give a positive reaction. The same reasoning *mutatis mutandis* applies to the newborn D-litter. By means of these two litters, the mutual reactivity of D- and R-cells in the chimaera is revealed, provided, of course, that D-cells are present in the chimaera.

TABLE IX.
Analysis of Chimaeric Reactivity

Test Litters	R < 24 h.	D < 24 h.	$(R \times T) F_1$ 4-10 day	$(D \times T) F_1$ 4-10 day
Injected Material	0 Chimaera Cells D Cells	0 Chimaera Cells R Cells	0 Chimaera Cells D Cells	0 Chimaera Cells R Cells
	pos. controls		neg. controls	
1.	+	+	+	+
2.	+	+	+	—
3.	+	+	—	+
4.	+	+	—	—
5.	+	—	+	+
6.	+	—	+	—
7	+	—	—	+
8	+	—	—	—
9.	—	+	+	+
10.	—	+	+	—
11	—	+	—	+
12.	—	+	—	—
13	—	—	+	+
14	—	—	+	—
15	—	—	—	+
16	—	—	—	—

The assay of competence to react against a third strain requires 2 hybrid test litters. These must be old enough to eliminate, through a host *vs.* graft reaction, that partner of the chimaera which is not accepted for genetical reasons, and hence, *it is that partner which is* allowed to display a graft *vs.* host reaction elicited by the T-antigens. Accordingly, as indicated in Table VIII, negative controls are now required in addition to non-injected litters. A positive reaction in the $(D \times T)$ hybrids is particularly informative if the supposedly chimaeric tissue fails to react in the R-litter. It rules out the possibility that the D-cells

failed to react, because of the fact that they were eliminated from the supposed chimaera

Analysis of chimaera 328/4: Recip. ST / Donor C_3H

Test litter: 24h C_3H

Spleen donor		Spleen weight mg/100g	Index
0	:	600	
Chimaera	:	650	1 08
Pos contr (ST):		1490	2 48

Conclusion: No Host vs Graft reaction

FIG. 3.

Analysis of chimaera 328/4: Recip. ST / Donor C_3H

Test litter: 24h $(C3H \times ST)F_1$

Spleen donor		Spleen weight mg/100g	Index
0	:	535 525 545 m: 535	
Chimaera	:	545	1 02
Pos contr (C_3H):		932 950	1 74 1 78

Conclusion: No Graft vs Host reaction if donor cells are at all present

FIG 4

The information provided by each of the 4 test litters can be expressed in terms of + or — reactions according to whether or not the injection of chimaera cells leads to increased indices of spleen and or liver Table IX also lists the 16 theoretical possibilities of combining and — reactions in 4 independent litters Of the 16 different patterns

nr 13 14 and 15 are the three which would be expected to meet the requirements of successful chimaera formation in clinical applications.

The figs 3-6 show for a single irradiation chimaera (328/4) the application of the principles outlined above.

Analysis of Chimaera 328/4: $\frac{\text{Recip ST}}{\text{Donor } C_3H}$

Test litter: 7-day (C_3H x DBA)F_1

Spleen donor		Spleen weight mg/100g	Index
0	:	635 595, m: 615	
Chimaera	:	1740	2 83
Neg contr (ST):		773	1 25

Conclusion: Strong Donor vs DBA reaction

FIG 5.

Analysis of chimaera 328/4: $\frac{\text{Recip. ST}}{\text{Donor } C_3H}$

Test litter: 9-day (ST x DBA)F_1

Spleen donor		Spleen weight mg/100g		Index	
0	:	329	256, m: 292		
Chimaera	:	334	390	1 15	1 24
Neg contr (C_3H):		364	350	1 25	1 20

Conclusion: No Host vs DBA reaction

FIG. 6.

One explanatory note is required Being short of a suitable R-strain litter (ST) we included instead a litter of F_1 hybrids between host and donor strains (C_3H × ST) This substitute served the purpose well in this particular case but would not always do so For example, if the donor litter had shown a + reaction no further information would be obtained from a + reaction in the (host × donor) F_1

failed to react because of the fact that they were eliminated from the supposed chimaera.

Analysis of chimaera 328/4: $\frac{\text{Recip. ST}}{\text{Donor } C_3H}$

Test litter: 24h C_3H

Spleen donor		Spleen weight mg/100g	Index
0	:	600	
Chimaera	:	650	1 08
Pos contr (ST):		1490	2 48

Conclusion: No Host vs Graft reaction.

FIG. 3

Analysis of chimaera 328/4: $\frac{\text{Recip. ST}}{\text{Donor } C_3H}$

Test litter: 24h $(C3H \times ST)F_1$

Spleen donor		Spleen weight mg/100g	Index
0	:	535 525 545 m: 535	
Chimaera	:	545	1 02
Pos contr (C_3H):		932 950	1 74 1 78

Conclusion: No Graft vs Host reaction if donor cells are at all present

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The information provided by each of the 4 test litters can be expressed in terms of + or — reactions according to whether or not the injection of chimaera cells leads to increased indices of spleen and/or liver Table IX also lists the 16 theoretical possibilities of combining + and — reactions in 4 independent litters. Of the 16 different pattern

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Analysis of Chimaera 328/4: $\frac{\text{Recip ST}}{\text{Donor } C_3H}$

Test litter: 7-day (C_3H x DBA)F_1

Spleen weight

Spleen donor mg/100g

0 : 635 595, m: 615

Chimaera : 1740 Index 2 83

Neg contr (ST): 773 1 25

Conclusion: Strong Donor vs DBA reaction

FIG 5.

Analysis of chimaera 328/4: $\frac{\text{Recip. ST}}{\text{Donor } C_3H}$

Test litter: 9-day (ST x DBA)F_1

Spleen weight

Spleen donor mg/100g

0 : 329 256, m: 292

Chimaera : 334 390 Index 1 15 1 24

Neg contr (C_3H): 364 350 1 25 1 20

Conclusion: No Host vs DBA reaction

FIG. 6.

One explanatory note is required Being short of a suitable R-strain litter (ST) we included instead a litter of F_1 hybrids between host and donor strains (C_3H × ST) This substitute served the purpose well in this particular case, but would not always do so. For example if the donor litter had shown a + reaction no further information would be obtained from a + reaction in the (host × donor) F_1

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Test litter: 24h C_3H

Spleen donor		Spleen weight mg/100g	
0	:	600	
			Index
Chimaera	:	650	1 08
Pos contr (ST):		1490	2 48

Conclusion: No Host vs Graft reaction

FIG. 3.

Analysis of chimaera 328/4: Recip. ST / Donor C_3H

Test litter: 24h $(C3H \times ST)F_1$

Spleen donor		Spleen weight mg/100g	
0	:	535 525 545 m: 535	
			Index
Chimaera	:	545	1 02
Pos contr (C_3H):		932 950	1 74 1 78

Conclusion: No Graft vs Host reaction if donor cells are at all present

FIG. 4

The information provided by each of the 4 test litters can be expressed in terms of + or — reactions according to whether or not the injection of chimaera cells leads to increased indices of spleen and/or liver. Table IX also lists the 16 theoretical possibilities of combining + and — reactions in 4 independent litters. Of the 16 different patterns

nr 13 14 and 15 are the three which would be expected to meet the requirements of successful chimaera formation in clinical applications.

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Test litter: 7-day $(C_3H \times DBA)F_1$

Spleen donor	Spleen weight mg/100g	Index
0 :	635 595, m: 615	
Chimaera :	1740	2 83
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Spleen donor	Spleen weight mg/100g	Index
0 :	329 256, m: 292	
Chimaera :	334 390	1 15 1 24
Neg contr (C_3H):	364 350	1 25 1 20

Conclusion: No Host vs DBA reaction

FIG 6.

One explanatory note is required Being short of a suitable R-strain litter (ST) we included instead a litter of F_1 hybrids between host and donor strains $(C_3H \times ST)$. This substitute served the purpose well in this particular case, but would not always do so. For example, if the donor litter had shown a + reaction no further information would be obtained from a + reaction in the (host × donor) F_1

The combined information of figs. 3 6 places the chimaera 328/4 in group nr 15 in Table IX i.e. one of the three desirable groups for *successful chimaera formation*

Up to the present a total of 4 irradiation chimaeras have been analyzed by this method. Table X gives the results obtained in terms of + or — reactions. It is seen that none are identical



The three $\frac{C_3H}{ST}$ have it in common that they produced a strong reaction in newborn ST litters (donor strain). In fact these reactions were significantly stronger than those produced by the positive controls. The conclusion therefore is clearly that in these 3 chimaeras recipient cells have not only recovered their ability to react against the donor strain but they have, moreover been rendered immune to it

TABLE X
Immunological Reactivity of Chimaera Spleen

R/D Chimaera	Test litters			
	R	D	(R × T) F	(D × T) F
C H / ST (295/3)	—	+	+	+
C H / ST (295/4)	—	+	+	—
C H / ST (294/5)	?			?
ST / C H (328/4)	—	—	—	

This host vs graft reaction may in two cases (295/4 and 294/5) have led to complete elimination of donor cell because there was no indication of cell being present which could react in either recipient strain or in the (D T) F Elimination of donor cell however is not the neces-

sary conclusion and an independent test for the presence of such cells would be needed for a full interpretation of the analysis performed in these two cases

In chimaera 295/3 on the other hand there is proof of persisting donor cells because of the + reaction with the (D × T) F_1 Furthermore, from the — reaction with the newborn R it can be inferred that the donor cells have lost, or never acquired, reactivity against the recipient.

All three of these $\frac{C_3H}{ST}$ chimaeras were survivors from the group in Table VIII which received repeated injections of foetal ST livers in a total dose of 70-77 mill. nucleated cells. They were sacrificed for analysis 3-3.5 months after irradiation at which time 294/5 showed some wasting and loose stools while the other two looked normal Post mortem showed nothing peculiar except that the spleens appeared to be roughly twice the normal size.

Though the number of properly analyzed chimaeras is so far small it seems no longer likely that secondary disease is always due to a graft vs. host reaction although the evidence by Trentin [16] and Uphoff [17] and others is incontestably suggestive. The truth is probably that host vs. graft, and graft vs. host reactions can both lead to pathological changes which are labelled as « secondary disease »

The more refined the methods become for analyzing the immunological state of chimaerism the more is likely to be revealed of the complexity of ways by which host and donor cells can influence each other It remains to be seen if all of the 16 different patterns of immunological behaviour outlined in Table VIII can in fact be realized under experimental conditions and not least, which factors favour one course of events over the others

These 16 possibilities far from exhaust the actual possibilities of variation. For example, a — reaction may reflect different kinds of non reactivity from something equivalent to acquired tolerance to a general exhaustion of immunological power

The procedure described in this section makes a very direct approach to the study of immunological reactivity in chimaeras. For analytical purposes, it seems more satisfactory than methods based on grafting of skin of tumours to the chimaera itself The latter methods may well reveal the outward reactivity of the chimaera (which is of course of the greatest importance) but need not throw light on the real state of affairs in the centres of immunological reactivity where host and donor cells are intimately mixed For example, a take of donor-type skin does not necessarily mean that the host is immunologically tolerant of D-antigens

It might also reflect a more or less permanent stale mate in the internal fight between r-e host and donor cells. The ultimate fate of the chimaera might be very different in the two situations

With the knowledge available today it seems both desirable and feasible that immunological studies of chimaerism in mice should account for the persistence of r-e cells of donor origin and also account for the immunological competence of donor and host cells separately not only in respect to antigens of each other but also in respect to antigens which are foreign to both

We are enclined to believe that among methods now available the *in vitro* cytolysis technique described elsewhere in this volume by P A Gorer is the most satisfactory for checking the persistence of donor cells. Furthermore we believe that the immunological competence of chimaera constituents perhaps can be assayed by the method described in this section

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DISCUSSION

WOODRUFF It seems to me that there is one other trick that one can play that might help this analysis. Apart from pre-immunising the donor of the cells that you are going to use, you can induce tolerance in the donor of the cells you are going to use. We are doing this in order to answer a slightly different question, namely can one have a tolerant cell as well as a tolerant animal?

SIMONSEN I perfectly agree that this should be tried.

LOUTIT Dr Simonsen's paper represents a very ingenious and a very beautiful method of testing the chimera but I'm not sure that the complicated analysis we have seen this afternoon necessarily solves all the problems of graft versus host or host versus graft reactions. For as Simonsen has pointed out, he has taken what is a new system for most of us, the 24 hour irradiation and the 1200 r dose to the mouse C3H. Now this may be the LD_{100} but we have still to show that this is the permanent inactivating dose for the reticulo-endothelial system or that part of the reticulo-endothelial system that forms lymphoid elements. And I think we should suspend judgement until Simonsen has a good deal of data on this dose with these strains

SIMONSEN Obviously the comment by Dr Loutit does not refer to the principles of analysis but rather to the conclusions which can be drawn from our actual irradiation experiments. And I perfectly agree that it has still to be established whether or not a low dose rate of lethal irradiation which has been used in our experiments is relatively less depressing on the antibody forming apparatus than on the hemato-poietic apparatus And this again could in theory at least, very easily be checked by applying the graft versus host assay this time not with chimera cells but with spleen cells from irradiated animals which are not subsequently grafted. And this I think should be done in comparing animals lethally irradiated with a low dose rate with others given a high dose rate. It may seem stupid that we have not done this already and I think it is, but it will soon be done.

OWEN I would like to comment briefly on the gene-dosage pheno-
nenon discussed by Dr Simonsen He will be pleased to know that
r Uphoff has in fact interpreted her data in just these terms her paper
believe has just appeared in the *J Nat Cancer Inst* The discussion
this subject should I believe, refer to the earlier work of Main and
hn who suggested that there was a dosage effect of just thi

in terms of skin graft survival The phenomenon of an allele dosage effect is a common one in blood group serology It may be worth mentioning however that because a heterozygote reacts more weakly than the homozygotes does, it does not necessarily follow that this is a simple gene dosage effect Ceppellini has established an interesting example in the field of Rh typing The heterozygote for any of the R alleles at the Rh locus with r (R/r') results in a weak and different anti Rh O reactivity known as « D » Now this might look like a dosage effect for components D/d but the fact is in this symbolism that the effect occurs when D is opposite *dC* (R/r') and *not* when it is opposite dc (R/r).

So there appears to be some kind of inter allelic interaction perhaps at some distance from the gene itself that results in this reduced reactivity It would be very interesting to apply your kind of analysis to the H 2 complex to see if there are comparable inter-allelic interactions on the basis of Dr Gorer s factorial symbols for the H 2

Blood transfusion in fowl
An example of immunological tolerance requiring the persistence of antigen

N. A. MITCHISON
Zoology Department, University of Edinburgh, Scotland

INTRODUCTION

Once properly established actively acquired tolerance of homologous tissue lasts a full life time. This has been shown particularly clearly by the blood chimaeras of man and cattle. It was pointed out by Owen [10] that the stability of the proportions of the mixture in the blood of cattle twins shows that there is no tendency for the tolerance to diminish in later life. The same stability is shown in experiments. Billingham, Brent, and Medawar [2] for example kept two tolerant mice bearing skin homografts for 450 days. The evidence for stable tolerance is less clear in animals with a longer interval between the initial inoculation and challenge. The mice of Billingham, Brent, and Medawar were left for 6-8 weeks after birth before challenge with a skin graft but there is no reason to suppose that fewer mice would have accepted their grafts had the challenge been delayed.

The effect of injection of soluble antigens into the embryonic or newborn animal is different. It is true that the earlier experiments appeared to show a long lasting tolerance after the injection of large quantities of human or bovine serum into newborn rabbits. But in the more recent experiments of Smith and Bridges [13] on rabbits, Wolfe, Tempelis, Mueller and Reibel [15] on chickens and Terres and Hughes [14] on mice the tolerance has turned out to be more transient. Both the duration and the degree of reduction of antibody production seem to depend upon the amount of antigen initially injected. The efficiency of heterologous tissue in eliciting long lasting tolerance is probably intermediate between homologous tissue and the soluble antigens. The un

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which to renew their own depots This assumption has received striking confirmation from the recent experiments of Billingham [1] These have shown that tolerant mice, when sufficiently carefully tested invariably turn out to be chimaeras in their lymph nodes. A ready explanation is also provided for the transience of tolerance to heterologous tissue the transplanted cells are unable to compete successfully in a foreign environment, and are gradually eliminated for non immunological reasons Something of this sort seems to take place when rat bone marrow is transplanted into irradiated mice (Ford Ilbery and Loutit [8]) The mechanism by which depots of antigen could inhibit the immunological reaction is discussed in a later section

Some evidence already suggests that the duration of tolerance depends on persistence of the antigen. The clearest comes from the experiments of Smith and Bridges [13] which have already been cited These have shown that rabbits which were injected with bovine serum albumin at birth and challenged several months later were less likely to react if they had already been challenged 14 times The earlier challenges evidently had the effect of prolonging the state of tolerance. Medawar and Woodruff [9] induced tolerance in rats by a skin homograft at birth excised it later and tested the tolerance afterwards. They found that the effect of the excision was to weaken but not abolish the tolerance.

The aim of the present experiments was to induce tolerance by anti genic stimulation for a controlled length of time. An antigen was ther efore chosen which is eliminated fairly rapidly from the body In this ay the time of stimulation could be varied by reinjecting the antigen at intervals Antigenic stimulation was continued well past birth, so as to ensure a maximum degree of tolerance. In an ideal experiment the persistence of antigen within tissue cells would be measured But since this is not possible, an antigen was chosen which could be detected in the blood. In this way it was possible to space the reinforcing injections so that some antigen was present in the body throughout the period of stimulation The animals were challenged with the antigen at an interval after the last reinforcing injection. In control animals the reinforcements were continued past this point in order to ensure that the state of tolerance was capable of lasting so long If the maintenance of tolerance depends on the retention of antigen the tolerance should vanish when the antigen is eliminated

Erythrocytes were chosen as the antigen. Donors and hosts could therefore be taken from the same species This is the relationship which is of greatest interest to the surgeon It has, in addition an important theoretical advantage, if we can extrapolate the argument of Humphrey [7] This is that homologous proteins which enter tissue cells are

published investigations of Hašek and Hraba on parabiosis of the eggs of chickens and turkeys has shown that the chickens which hatch even though they may contain turkey blood and haemopoietic cells for months always lose these cells eventually

The hypotheses which have been put forward to account for the duration of tolerance fall into two categories One category assumes that a heritable change takes place in the antibody producing cells of the tolerant animal On this assumption the persistence of antigen is not vital for the maintenance of tolerance This, on the whole is the view which has been most favoured by workers who have been concerned with the transplantation of tissue (Brent [4]) Various versions of it have been discussed A less sophisticated version assumes that the initial antigenic stimulus induces some change in the genetic machinery of the cell—possibly in the genes, but not necessarily This change is then transmitted from the affected cells to their progeny It is clear that the change must be heritable for new cells are being formed all the time in the lymph nodes and spleen and unless these cells are also non reactive, the tolerance would be broken It seems to me that the more we learn about the genetics of cells, the less probable is this hypothesis For it assumes new genetic information can be created by the stimulus of the antigen Professor L. Szilard (personal communication) has pointed out that this difficulty can be overcome by assuming that the antigen breaks genes which are already present

In a more sophisticated version it is assumed that living cells when they induct tolerance actually transfer (or transduce) some of their own genetic machinery into the cells of the host This certainly provides an explanation for the more transient effects of soluble antigens—indeed these would have to work by some different mechanism But again it seems to me that we have little reason to think that somatic cells are so ready to indulge in sex (or the substitutes for sex or vegetative hybridisation).

The persistence of antigen is vital for the maintenance of tolerance according to the alternative hypotheses This is the view which has been favoured by those who have worked with soluble antigens (Smith and Bridges [1]). The fact that the tolerance induced by soluble antigens is long lasting even though not permanent can be readily understood For there is excellent evidence that antigens such as bovine serum albumin can be retained in tissue cells for months at least—although this evidence applies so far only to situations in which the animals are immune not tolerant Tolerance to tissue is assumed to be permanent because the animals are chimaeras The antibody producing cells are thus provided with a permanent source of antigen from

which to renew their own depots. This assumption has received striking confirmation from the recent experiments of Billingham[1] These have shown that tolerant mice, when sufficiently carefully tested invariably turn out to be chimaeras in their lymph nodes. A ready explanation is also provided for the transience of tolerance to heterologous tissue the transplanted cells are unable to compete successfully in a foreign environment, and are gradually eliminated for non immunological reasons Something of this sort seems to take place when rat bone marrow is transplanted into irradiated mice (Ford Ilbery and Loutit[4]) The mechanism by which depots of antigen could inhibit the immunological reaction is discussed in a later section

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quickly degraded while foreign proteins are held for long periods In consequence, the depots of antigen which are held outside the circulation will be exhausted more quickly if the antigen is homologous.

Erythrocytes have a limited life time. But it is essential that they should not be contaminated with living white cells for these would be expected to induce a long lasting state of tolerance. This aim was not completely achieved in the present experiments, where heating was the method used. However the contamination seems to have been a minor one which did not interfere with the main investigation For this reason the quantitative results of the present experiments should be treated with reserve. The fate of the erythrocytes after transfusion was followed by labelling them with radioactive chromium 51 according to the standard clinical procedure.

Birds where used as the experimental animal They were first chosen because of the ease with which blood can be repeatedly withdrawn and injected into embryos and adults. The choice proved to be a fortunate one for in preliminary experiments with mice the elimination of homologous erythrocytes was found to be much delayed

Blood transfusion in fowl.

Blood for transfusion was withdrawn from the wing vein into a syringe containing 0.1 vols. of a solution of heparin in physiological saline containing 100 i.u./ml. It was then incubated for 1 hour at room temperature with chromium 51 as sodium chromate at a concentration of 10-30 μc/ml whole blood (obtained from the Radiochemical Centre Amersham) The concentration of chromium did not exceed 10 μg/ml blood No attempt was made to separate the cells from the radioactive plasma the blood was either injected directly after labelling or after subsequent treatment to kill leucocytes

The result of blood transfusion in normal adult fowl is shown by the following experiment with four adult brown Leghorn hens One bird was bled and then *auto*-transfused with its own labelled blood A second bird was transfused with blood from another brown Leghorn thus receiving an *intrastrain* tran fusion The remaining two each received a transfusion from a white Leghorn donor an *interstrain* transfusion The last three transfusions were repeated with the identical donors and hosts 23 days later The outcome of these transfusions are shown in fig 1 In this and subsequent figures (except fig 2) each line represents a single bird and each point a single sample of blood The broken line represents diagrammatically the injection of blood The percentage survival of the transfused blood has been calculated on the

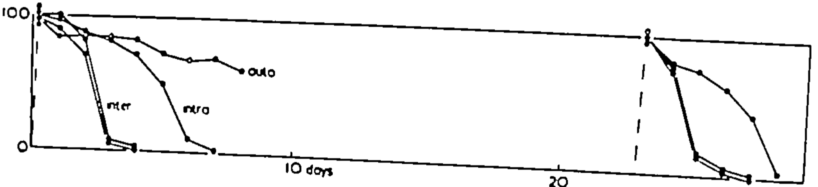
assumption that the first sample after transfusion constitutes 100 % after substraction of the radioactivity of the supernatant. Later samples are calculated as a percentage of the first sample, with allowance made for radioactive decay of the chromium 51. The auto-transfused blood exhibits precisely the same curve of elimination as that of blood auto-labelled by the injection of chromium-51 into the whole animal, as reported by Rodman, Ebaugh and Fox[11]. The auto-labelled blood in their experiments was eliminated with the normal tendency towards an exponential curve, attributed to the elution of the label from the circulating cells. The life of the erythrocyte in hens is reported to be 30 days. Within the limits of experimental error the foreign transfusions resemble the auto-transfusions to begin with. Then after 3-4 days with intrastrain transfusion and 1-2 with interstrain transfusion a phase of accelerated elimination takes place. There may be a final phase of slower elimination for unknown reasons. This pattern, of a relatively short survival followed by a fast phase, together with a slow phase of elimination is characteristic of the fate of transfused foreign blood. It is used in the subsequent experiments as the meth[od] of distinguishing the immunological reaction to transfused blood.

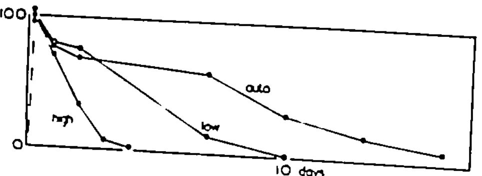
The choice of the curve of elimination from the blood instead o[f] the production of agglutinins may seem curious. A cursory search for agglutinins was carried out without success although they appear promptly in hens after the injection and elimination from the circula[-]tion of turkey erythrocytes. Justification for regarding the curve of elimination as characteristic of an immunological reaction can be found in its similarity to the curves for other immunological reactions such as bovine serum albumin in the rabbits. Furthermore the secondary response shown in fig. 1 is accelerated in the characteristic way by about one day.

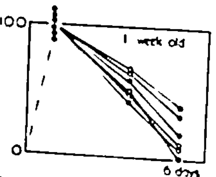
The immunological reaction is detectable 2-3 days earlier after interstrain than intrastrain transfusion. This combination has the additional advantage that variation is expected to be less. For these reasons the combination of white Leghorn donors and brown Leghorn hosts was used in later experiments.

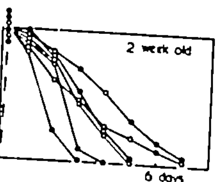
HEAT TREATMENT OF BLOOD

Following the lead of Billingham, Brent, and Medawar, heating [w]as chosen first as the method of killing leucocytes in blood. The effect [o]n the survival of erythrocytes was first investigated by heating to two [le]vels. Auto-transfusions were carried out on 5 chickens aged 2 weeks. [Th]e blood was stood in small glass bottles in a stirred water bath for









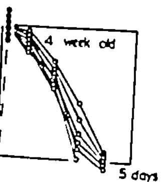


FIG. 1. Transfusion in adult fowl. Auto (white circles) auto-transfusion. Intra (half-black circles) brown Leghorn intra-strain transfusion. Inter (black circles) interstrain transfusions from white Leghorn donors to brown Leghorn hosts.

FIG. 2. Transfusion of heated blood. Auto (half black circles) auto-labelled controls. Low (white circles) blood heated to 48.3° C. High (black circles) blood heated to 49.2–49.9° C.

FIG. 3. Transfusions into chickens of three ages.

20 minutes after labelling and before injection Under these conditions the liquid in the bottles reaches equilibrium in 5 minutes at a level approximately 0 5°C below the temperature of the bath. Making allowance for this three of the samples were at 47 8 48 3 C for 15 minutes and the other two at 49 2 49.9 C. In addition four chickens of the same age were auto-labelled by direct injection of chromium-51 The average curves of elimination of each group are shown in fig 2 Heating at the lower level produces an appreciable effect on the survival of erythrocytes, while an increase of approximately 1 5 C cuts the life span to about 3 days. The margin is slight between the heat which renders erythrocytes unusable, and the heat which could be expected to kill leucocytes. As the later experiments turned out, this margin hardly exists at all. Nevertheless a series of experiments were then begun using the lower temperature level.

The reaction of chickens to homologous erythrocytes

The age at which chickens mature immunologically was next investigated The unpublished experiments of Miss Ann Beckitt on the elimination of turkey erythrocytes by young chickens were used as a guide. She has found that newly-hatched chickens do not react immunologically as judged by the curves of elimination the reaction is first shown on the fifth day after hatching Homologous blood was accordingly transfused into four groups of chickens, aged 0 days 1 2 and 4 weeks The blood was heated as before to 48.0 48 5 C for 15 minutes before transfusion The elimination of the transfused cells in the three older groups of birds are shown in fig 3 There was little sign of an immunological reaction in the group aged 1 week In the group aged 2 weeks, two birds reacted definitely and a further two may have a delayed reaction The reactions of the group aged 4 weeks are more uniform all exhibited the accelerated phase of elimination by the third day after transfusion These three groups have thus spanned the immunological maturation and we shall be safe in assuming that chickens are mature in their immunological reaction to homologous erythrocytes at 4 weeks of age

Induction and maintenance of tolerance by heated erythrocytes

Four groups of chickens were subjected to a course of transfusions of heated homologous blood designed to induce and maintain a state of tolerance Each group received transfusions from a single white Leg

horn hen. The donors appeared to suffer no ill-effects from being repeatedly bled one went on laying eggs through the whole experiment Two of the groups began their course as « newly hatched chicks supplied by a dealer but were in fact aged between 2 and 48 hours at the time. Three birds from each of these two groups were carried through the experiment as shown in figures 4 and 5 though these groups began with nine and sixteen birds respectively The curves of elimination varied markedly between individuals during the first two weeks and only the slow eliminators were kept. The source of this variation has not been entirely explained Some of it was presumably due to the range of age at first transfusion but occasional individuals in other experiments have shown a consistent pattern of accelerated elimination which begins in the egg and is repeated after successive transfusions. The other two groups were first injected in the egg on the 17th day of incubation Three birds from one group and four from the other were carried through the experiment as shown in figures 6 and 7 these groups started from fourteen and twelve hatched birds respectively The remaining birds in these two groups died within two months of hatching

Several signs of an immunological reaction of transplanted cells against their host could be recognised in the birds which died Death was preceded by anaemia and failure to put on weight and the spleen was often enlarged and haemorrhagic at autopsy On the other hand the birds died later than would have been expected from transfusion of unheated blood. Simonsen [13] transfused embryos with homologous blood and found that the majority of the deaths occurred during the first fortnight after hatching: this was confirmed with the present material. The majority of the deaths after transfusion of heated blood (14/19) occurred later The most likely explanation of these deaths is that a small number of leucocytes survived the heat treatment The fluctuation of the temperature of the water bath during the whole experiment was in the range 47.9 49.2 C. It was not expected that leucocytes would survive exposure to this temperature even though the normal temperature of birds is higher than mammals.

The aim of the course of transfusions was to maintain a detectable number of homologous erythrocytes in the circulation On the whole this aim was achieved or at least the periods of absence of homologous cells were limited to points on the last day of the curves of elimination Sometimes (for my own convenience) a transfusion was given before the preceding one had been much eliminated. The shape of the curves varied particularly during the first day After the first day the shape tended to resemble an inverted S with a period of slightly faster eli

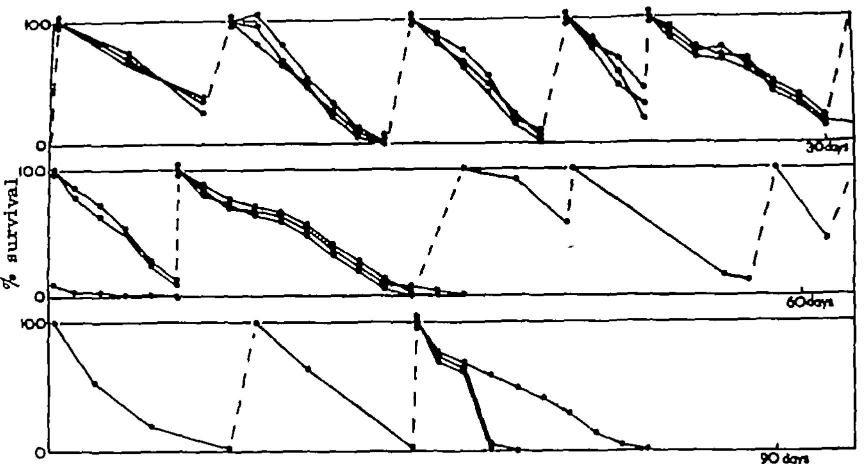


FIG. 4. — Induction and maintenance of tolerance by heated blood. Course of transfusions starting in « newly hatched » chickens. White half black, and black circles indicate different individuals.

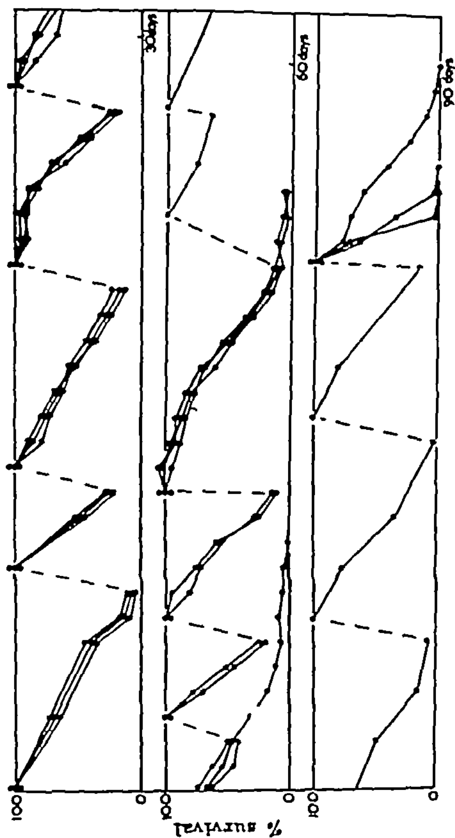


Fig. 5 — As figure 4 another series.

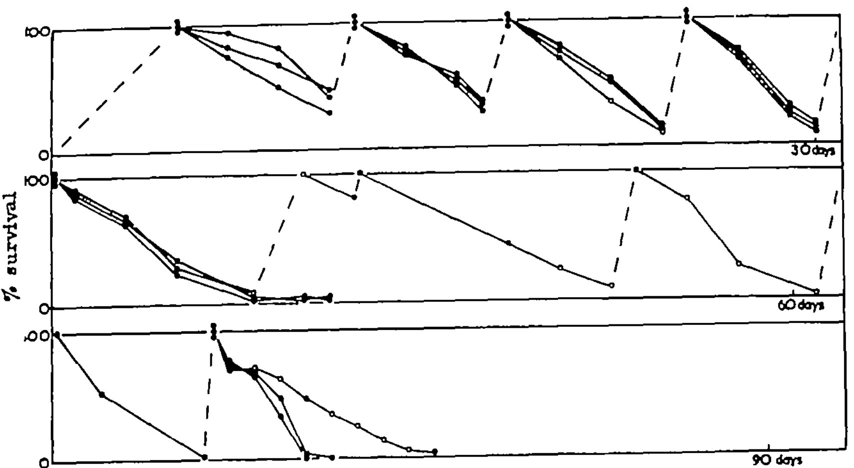


FIG. 6. — As figure 4, but transfusion start in egg.

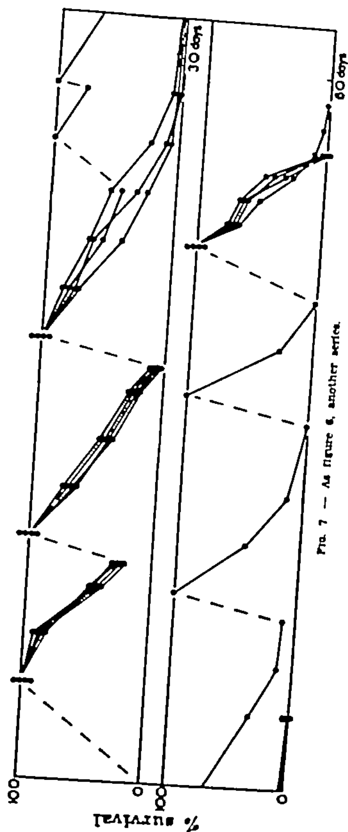


FIG. 7 — As figure 6, another series.

mination between 4 and 7 days after transfusion, and a flat tail later. Unless the chromium is eluted more rapidly after heat treatment, the shape of the curve must mainly reflect variation in the fragility of the transfused cells. No signs of reincorporation of the chromium were expected and little were found: the prolonged tails after the 5th transfusion in figures 4 and 5 may possibly be due to reincorporation.

Turning now to a comparison at different ages, the curves of elimination do not change significantly. Except possibly in the group shown in fig. 7, there is no tendency for the rate of elimination to accelerate as the birds mature, nor does a phase of rapid elimination appear. The rate of elimination does vary from one transfusion to another, probably because of fluctuations of the temperature at which the blood was heated. The second transfusion in fig. 5 was eliminated exceptionally fast: this was the only blood which has been stored at 4° C before transfusion. The longest period for which homologous blood was maintained in the circulation was 90 days, as shown in fig. 5. The conclusion to be drawn from the constancy of the curve of elimination is that the transfusions have induced a state of tolerance.

The state of tolerance could be terminated by allowing the homologous blood to be completely eliminated. Eleven separate attempts to do this were made, of which we may first consider the 6th transfusions in the first two groups (figures 4 and 5). Only two of the birds in each group were transfused, while the remaining one preceded to eliminate the remaining homologous blood. After the remaining birds had spent 1-2 days without detectable homologous blood they were retransfused together with their fellows. This brief interval had no effect on their subsequent curves of elimination. The same procedure was repeated later but with a longer interval. Only one bird in each group was maintained after the 7th (fig. 4), 8th (fig. 5), 5th (fig. 6) and 3rd (fig. 7) transfusions. After homologous blood had not been detected in the remaining birds for 25-29 days, all were again transfused together. In one of the groups (fig. 7) the bird which had been maintained on homologous blood appeared to have lost most of its tolerance—perhaps because the maintaining transfusions had been too widely spaced. The results were clear-cut in the other three groups. All of the birds which had been maintained on homologous blood were still tolerant. All the birds in which the transfusions had lapsed showed the sign characteristic of an immunological reaction: a delayed phase of rapid elimination.

Discussion

(i) *The presence of antigen as an essential for the maintenance of tolerance*

The conclusion may be drawn from these experiments that homologous erythrocytes can induce and maintain a state of tolerance. This tolerance can be abolished at any time by depriving the birds of erythrocytes. The point of abolition is reached between 2 and 25 days after the last homologous erythrocyte has left the blood. It does not follow that the erythrocytes in the blood are themselves responsible for the tolerance. It is more likely that a depot of intracellular antigen is essential and that this depot can be replenished from the cells in the blood. But before accepting these conclusions certain possible objections may be considered.

a) *Degrees of tolerance.* Repeated doses of antigen given to the very young animal probably provoke a higher degree of tolerance than a single dose of the same total amount. Repetition for example may stimulate a larger number of lymphoid cells. This phenomenon is entirely distinct from the maintenance of tolerance. All the birds in the present experiment received the same dosage of antigen until after their normal age of immunological maturity.

b) *Survival of erythrocytes.* The curves of elimination may not be comparable when some birds have homologous erythrocytes already present before transfusion and others do not. Thus the erythrocytes which are already present may constitute a selected population of less fragile cells. This is probably the case. But it will not explain the difference between the birds which had been maintained on transfusions and those which had lapsed for the longer interval. For the shorter lapse had allowed all trace of the homologous erythrocytes to disappear yet was without significant effect on the subsequent curve of elimination.

c) *The effects of radiation.* The total dose of radiation received by each bird during the whole course of transfusion is calculated at less than 10 r. This is hardly sufficient to interfere with the immunological reaction.

d) *The persistence of cells is not required for the suppression of agglutinin.* Hašek and his colleagues (Hašek) have shown that transfusion of foreign blood into young chickens causes a long lasting suppression of agglutinin production. Billingham, Brent and Medawar[1] were

able to show that this capacity is not abolished by heating the blood to 49–50° C before transfusion. This transient stimulus can therefore induce a long lasting state of tolerance. The discrepancy with the present results can be accounted for by assuming that the curve of elimination is a more delicate test for an immunological reaction. In confirmation of this assumption Owen[16] has shown that chickens which have been injected with heterologous blood at hatching tend to produce incomplete antibodies rather than agglutinins.

e) *Contamination with killed leucocytes.* Each transfusion of blood must have contained a large number of killed leucocytes. The possibility has not been excluded that their antigens were responsible for the observed phenomena. However, this possibility does not interfere with the principal conclusion that the presence of antigen is required for the maintenance of tolerance.

(ii) *A possible practical application*

My colleague, Professor M. F. A. Woodruff, has pointed out the following possibility. Human patients may sometime be lethally irradiated and then inoculated with homologous bone marrow. Foetal donors would be used in order to avoid the risks of a reaction of the graft against the host. But the same risk would be encountered again if the patients were transfused with blood from adult donors during recovery. A stringently tested method of killing leucocytes in blood without damaging erythrocytes, would then be a valuable weapon for the surgeon. Even though heating is clearly not to be recommended other procedures can and will be tried out with the present system.

(iii) *Cellular mechanisms*

Let us consider three simple hypotheses each of which assumes that the persistence of antigen is required for the maintenance of tolerance. According to the first, the antibody producing cells are working in the normal way in the animal which is apparently tolerant. But so much antigen is present in the body that all the antibody is absorbed and eliminated immediately after synthesis. Something of this sort may take place during immunological paralysis by polysaccharide. This tread-mill hypothesis cannot account for immunological tolerance to homologous tissue nor to bovine serum albumin, for in both cases the tolerance can be abolished by the transplantation of lymphoid cells into the tolerant animal (Billingham, Brent, and Medawar[3]; Smith and Bridges[19]). The evidence against it in the present experiments is that no acceleration in the rate of elimination of the foreign erythrocytes was detected as the birds matured.

According to the second hypothesis, the presence of antigen in a cell is required for antibody production to take place. We assume that there are two sites available to an antigen in the antibody producing cell. If the first is occupied, antibody is made. If the second is occupied then the first is blocked. It is the second site which is occupied in the tolerant animal. Of course the two sites might equally well be two positions on a single site. Both sites can hold more than one molecule of antigen and this reserve can be filled up from the circulation as it is exhausted. But the tolerance site is open only in the young animal; in the mature animal it can be filled up as it empties, but once empty it is closed forever.

The third hypothesis has been put forward by Professor J. Lederburg[8]. Antibody production is assumed to be due to selection within a population of cells, which is divided into clones which make specific antibodies. These clones are absent from the embryo and originate by *transitions* from undifferentiated cells occurring at intervals in later life. During transition to later life as a producer of specific antibody the cell passes through a stage when it can be killed by the specific antigen. In this way if antigen is already present it will inhibit the production of the cells which make antibody against it.

Thus the analysis of tolerance is bound up with the analysis of immunity. The evidence so far is compatible with both the last two hypotheses. It is possible that the present experimental approach may help. On Lederburg's hypothesis, we predict that tolerance should begin to diminish very shortly after the antigen is eliminated and should do so gradually and irregularly. This is not expected otherwise when we would expect the entire population of antibody producing cells to be able to participate in the immune reaction as soon as they have exhausted their reserve of inhibitory antigen. Unfortunately we do not know enough to be able to make these predictions quantitative.

SUMMARY

A method is described for following the elimination of transfused erythrocytes in fowl by means of radioactive chromium. The curve of elimination, as determined in this way can be used to recognise the immunological response to homologous blood. Immunological tolerance could be induced by transfusion of heated blood and maintained by retransfusion at intervals. The tolerance could be abolished by allowing the transfusions to lapse. The conclusion is drawn that the presence of antigen is required for the maintenance of tolerance in this system.

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DISCUSSION

MEDAWAR. Mitchison has established the important theorem that the persistence of tolerance in the whole animal is a function of the persistence of antigen, tolerance disappearing when (or not long after) antigen disappears. This means that we shall have to redefine tolerance in some such terms as

« If an animal is exposed to an antigen before it has developed the capacity to react against it then the development of that capacity is delayed and under certain circumstances, may be indefinitely postponed »

The emphasis of the definition now rests wholly on the time of origin in development of the power to undertake a specific immunological response.

I question Mitchison's tentative inference that the need for antigen to persist tells against an « adaptive » theory of tolerance. Surely we must first distinguish between two possibilities

(A) Cells capable of being made tolerant occur *only* in embryos or immature animals

(B) Immunological stem cells persist throughout life and during maturation pass through a state in which they are capable of being made tolerant

If (A) is true then the results of Mitchison and of Smith and Bridges show that persistence of antigen is necessary to maintain tolerance in the cell or cell-lineage in which tolerance was induced. But if (B) is true then persistent antigenic stimulation may be needed not to

According to the second hypothesis, the presence of antigen in a cell is required for antibody production to take place. We assume that there are two sites available to an antigen in the antibody producing cell. If the first is occupied antibody is made. If the second is occupied, then the first is blocked. It is the second site which is occupied in the tolerant animal. Of course the two sites might equally well be two positions on a single site. Both sites can hold more than one molecule of antigen and thus reserve can be filled up from the circulation as it is exhausted. But the tolerance site is open only in the young animal in the mature animal it can be filled up as it empties, but once empty it is closed forever

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maintain tolerance in cells or lineages already tolerant but to *induce* tolerance in the antibody forming cells which are maturing throughout life. Perhaps antigen serves both purposes.

There are certain other consequences of hypothesis (B) One of them is that if an adult animal were so treated that all *mature* antibody-forming cells were destroyed exposure to antigen should induce a state of tolerance. Can this be, as Loutit has suggested the explanation of radiation induced tolerance?

A second consequence is this. As Mitchison says, there are broadly speaking two conceptions of how tolerance comes about

(C) *Tolerance is due to the actual death of a cell clone pre-adapted* to making a particular kind of antibody

(D) Tolerance involves some change in a cell and its derived lineage *other* than its destruction

Possibly (C), as Mitchison points out, seems to be required by certain modern variants of the « clonal predestination » theory of anti body formation Possibility (D) taken in conjunction with (B) leads to a rather unexpected inference viz that although the effect of anti genic stimulation in very early life is to induce tolerance, the effect of antigenic stimulation on the adult is to produce both tolerance and immunity for when an adult is exposed to antigen the mature body forming cells will react by antibody formation while the immature cells envisaged by hypothesis (B) will be made tolerant This interpretation has the further necessary consequence that no antigenic stimulus, however power ful or however often administered can fully saturate the antibody forming system each stimulus must automatically produce a crop of cells which are tolerant of that antigen and which are thus released for react ing against some other antigen. This would explain one of the facts that is particularly well accounted for by the clonal predestination hypothesis viz that after antigenic stimulation not all cells are engaged *in the specific immunological response* (This explanation is not com plete because we should still need some rationing system to ensure that when an animal is exposed to antigens I and J simultaneously some *cells react to I and others to J*).

HAŠEK I am not sure that in chickens, it really is more profitable to use the disappearance of antigen as an indication of an immune response Did you measure the titre of agglutinins?

MITCHISON Yes but sporadically After a single injection of anti gen even in non-tolerant birds I think it is unusual to find agglutinins.

HAŠEK Perhaps that does not apply to a heterologous system?

MITCHISON That is correct Agglutinins appear regularly after the immune elimination of turkey erythrocytes.

HAŠEK We find that when we inject soluble antigens—for example albumins—the secondary response is much less distinct than in rabbits there is a difference of one day I think we agree about this.

Another point is that for killing cells—leucocytes—it might be useful to use some effective but not grossly damaging dose of irradiation I think 2000 r should certainly be sufficient As to the evidence of persistence of antigen, I completely agree with your results but perhaps a quantitatively different mechanism may be at work in this kind of erythrocyte tolerance. In parabionts, we have found by absorption techniques that one year after we can no longer detect chimerism in erythrocytes, we still have a complete suppression of agglutinin formation

HAŠKOVA I agree with Dr Mitchison's main conclusions and I should like to present one from our experience with heterologous systems. We injected newborn ducklings with a certain quantity of goose blood and after immunization with the same blood 8 weeks after hatching we found complete suppression of agglutinin formation. During these experiments, we found no erythrocyte chimerism at all. It has also been our experience that heterografts of goose skin in ducks never survive for more than 29 days. After immunizing the ducks which were negative at eight weeks for a second time 33 weeks after hatching we again found complete suppression of heteroagglutinin formation So I think it is possible that there is some persistence of antigen in the recipient's cells—presumably the donor cells disappeared just as the skin heterograft did.

BRENT The suggestion by Medawar that tolerance may be due to an effect on immature stem cells appeals to me greatly but I am not quite with him in his final conclusion On his hypothesis, it should be possible to render an adult animal tolerant by the maintenance of an antigenic stimulus over a sufficiently long period of time for the animal's lymphoid cell population should with time, be transformed from one consisting predominantly of cells capable of the specific immune response into one in which *all* or nearly all cells had been made tolerant of the antigen in question The withdrawal of the antigen would permit the formation of new non-tolerant cells and so to a breakdown in tolerance.

MEDAWAR We may have to introduce some subsidiary hypothesis to meet Brent's point—possibly the fact that antigen may preferentially

stimulate the division of the mature cells engaged in making antibody against it as Coons's work suggests.

SIMONSEN I must say that the theory which Professor Medawar has just called the « stem-cell » theory appeals to me very much. It was this kind of thing which I meant when I spoke earlier about immunological selection pressure on adult cells transplanted into an F_1 hybrid, the donor being one of the parental strains (D) The decline of the spleen and of the liver indices in this combination which come down to normal after a month or so provides the best system I can devise for testing the theory For you can make a graft versus-host assay with these spleens which are presumably chimeric in a test litter of constitution (D × T) F_1 where T is a third strain.

In this test a positive reaction could mean that the immature cells from the original adult donor (D) had become non-reactive at least, if not tolerant towards their first host while retaining their general immunological potentiality

WOODRUFF There are two comments on Professor Medawar's hypothesis I would like to make. The first is a question. Has anybody ever demonstrated that there are such things as tolerant cells? We feel there are, but it seems to me highly important that it should be shown, and as I mentioned in discussing Dr Simonsen's paper we are at present doing an experiment which we hope will provide an answer

My second comment is this. An awkward fact has been brought to light by a clinical experiment of Dr Linden Peer's in which he showed that not only do grafts of skin from mother to child occasionally survive for an unexpectedly long time (which can be explained as a maternally induced tolerance) but sometimes the reverse happens, and grafts from child to mother though never from child to father also do just this. I was a little sceptical about this until Dr Peer very kindly allowed me to examine some of his patients but now I accept it as a fact It has occurred to me that this postulated curious state of affairs in which an abnormally large number of cells capable of being made to respond to an antigen by becoming tolerant instead of responding by becoming immune, might possibly occur during pregnancy and in this event Peer's observation could be accounted for by entry of fœtal cells into the maternal circulation. It would be extremely interesting to see whether you could make a pregnant mother tolerant to some quite unrelated homologous tissue antigens by exposing her to these antigens while pregnancy was in progress.

MEDAWAR: I quite agree with Woodruff's first point. No-one, I think has demonstrated that there is such a thing as a tolerant cell or a tolerant cell lineage. It should not be very difficult to find out and we all hope that Professor Woodruff will do so.

With regard to the second point: there are various kinds of possible explanations of Peer's rather curious result viz the rather long survival time of homografts transplanted from child to mother as opposed to the survival time of homografts transplanted from child to father. There is the remote possibility that, inasmuch as there seems to be some evidence that foetal cells of various kinds can enter the mother something comparable to «enhancement» might occur. I am afraid that at this Colloquium we are using enhancement as a cure for all evils, so I hesitate to press that point, but it is just a possibility.

WOODRUFF: Yes, I did mean the notion of foetal cells entering the mother but the mother treating them in this peculiar way because she was for the time being pregnant.

BERRIAN: Professor Medawar's hypothesis describes a situation that may exist in adult animals as compared with immature ones. It does not seem relevant to the nature of the alteration within stem cells capable of tolerance that somehow enables them to manufacture antibody. That is the nucleus of the problem. The hypothesis has however restated the situation in an interesting and thought provoking manner. Is my interpretation correct or have I missed some point accounting for the maturation of antibody forming cells?

MEDAWAR: No what Dr Berrian says is perfectly correct. The few remarks I had to make had no bearing whatsoever either on the process of maturation from a stem cell to a mature cell, or upon the question of how it is that a stem-cell or an embryonic cell becomes tolerant.

Erythrocyte repopulation after transplantation of homologous erythropoietic tissues into irradiated mice

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Introduction

Transplantation of blood forming tissues into heavily irradiated recipients has become a popular subject of study in recent years partly because of the potential utility that may be foreseen for such transplants, and partly because the system brings into focus a variety of fundamental problems relating to the general subject of tissue transplantation No attempt will be made in this brief paper to review or cite completely the already very extensive literature in this area A few specific citations will be made at points where documentation seems desirable most of the references cited review the relevant literature.

Recognition that cellular transplants may become established in heavily irradiated recipients, in combinations of donors and hosts in which histocompatibility barriers would have prevented successful transplantation to unirradiated recipients has followed the application of several different kinds of markers for the distinction of donor from host cells. Intraspecies (homologous) combinations have been distinguished in rats by serological markers ( ) and in mice by an aberration in structure that permits chromosomes of the donor to be differen

( ) The work reported here was done while the author was a Research Participant on leave from California Institute of Technology Pasadena during 1957-58 Warm gratitude is expressed for the contributions of concept, work, and material to these studies particularly by Drs T Makinodan H Gengozian C C Congdon, I. C. Shekarchi, J W Goodman, R A Popp and G E Cosgrove and by Mrs. L Urso Acknowledgment is also due the Anna Fuller Fund for a grant in support of the author's travel to the Colloquium

( ) Operated by Union Carbide Corporation for the U S Atomic Energy Commission

tuted from those of the recipient[3]. Sex chromatin differences have been observed to distinguish cells deriving from the graft from those of the host when homologous donors and recipients differ in sex[19]. Recently inherited individual differences in hemoglobin have also provided markers of particular promise for homologous mouse combinations in which neither serological nor chromosomal analysis can be applied[19, 21]. The functional and morphological normality of blood forming tissues implanted into irradiated genetically anemic mice has provided another means of identifying successful transplants[21]. Tests for the antigenic character of repopulated tissues in terms of their capacity to immunize for « second-set » transplant rejection responses, have also served to identify the origins of these tissues[18]. The transplantation specificities of tumors that have arisen in treated recipients have been applied to good effect[7]. Indirect evidences of the persistence of a transplant have been derived from transplantation of skin having donor specificity to the treated recipients, both in homologous[19, 22, 26] and heterologous[26] combinations. Heterologous combinations particularly rat-into-mouse, have been studied with serological markers and enzymatic differences distinguishing the species[11, 18]. Other protein and certain physiological differences between donor and host cells (for example, differences in osmotic fragility[4, 11]) have also been applied.

Because the mouse has been used extensively for studies of this sort of transplantation, and because inherited diversity in mouse red cells is recognizable by serological methods[1, 6], it seemed that an application of serological distinctions would be fruitful for defining and following transplantation of homologous erythropoietic tissues in irradiated mice. The present discussion is intended to outline briefly the results of studies to date along this line.

Materials and methods

Antisera were prepared by injecting saline suspensions of cells of normal tissues (liver and spleen and frequently kidney and testis) from particular strains of mice intraperitoneally into mice of other strains. Three injections were given at weekly intervals, and the recipients were bled for serum about 10 days after the final injection. Several combinations of mice were immunized by injecting donor tissues in an adjuvant, by the procedure described by Munoz[14]. The ascites fluid that developed in quantity in several of the recipient lines contained antibodies in usable titer.

Tests of two sorts were performed. The dextran-human serum agglutination technique of Gorer and his associates[1] gave only rough quantitative estimates of the relative proportions of positive and nega-

tive cells in chimeras and we could not identify with certainty mixtures in which positive cells constituted less than 20 or 30 % of the total red cell population. In the second test procedure, a lytic system described by Hildemann [3] and somewhat modified for our purposes [11] each test tube held 0.2 ml of the proper dilution of the antiserum (relatively low antiserum dilutions of 1 16 or 1 32 were commonly used to achieve maximal hemolysis), 0.1 ml of a 2 % suspension of washed red cells, and 1 drop of fresh or fresh-frozen rabbit serum The rabbit serum had been absorbed briefly at a temperature near 0 C with about half its volume of washed packed pooled mouse red cells to remove normal rabbit-antimouse antibody from this source of complement. The tests were read after 2 hours at 37 C, the supernatant having been transferred after centrifugation to cuvettes for reading in a Beckman spectrophotometer at 578 mμ in a total volume of 3 ml. In the studies reported here, all dilutions and cell suspensions in the lytic test system were made with buffered saline containing added magnesium ion, as recommended by Hildemann. More recently we have diluted instead with the barbital buffer and added magnesium recommended by Pillemer [18] in his studies of complement and properdin

The lytic system permits an absolute measurement of the proportion *of red cells hemolyzed by antibody and complement. After subtraction* of a correction factor obtained from the supernatant of tests in which normal serum is substituted for antiserum performed in parallel in *each test on each cell suspension color caused by specific hemolysis can* be compared with the color deriving from total osmotic hemolysis of a similar test cell suspension in distilled water This « proportion of cells *specifically hemolyzed » for a chimera under test can then be compared* with (a) a similar value for normal animals known to be positive to the test reagent or (b) mixtures of known positive and negative cell suspensions. *Under proper* test conditions, the obvious calculations permit us to evaluate with considerable confidence intermixtures in which the proportion of positive cells ranges from about 5 to 100 % Care must be taken however that the concentration of none of the reagents is limiting with reference to the total number of cells to be lysed In our experience many antisera that were entirely adequate for qualitative application of the agglutination test have been inadequate for quantitative tests with the lytic system

Estimates with the lytic system must be recognized as statistics subject *to considerable variation. Part of this variation must* be in the technique itself but a significant part of it also reflects true biological variation. Even within an inbred line and in the same animal from one bleeding to another the proportion of cells lysable by particular

reagents varies. An idea of the extent of this variation may be gained from the following sample data taken at a time of rapid repopulation and maximal individual variability In experiment OR 18 ten days after the injection of (L × A)F_1 male bone marrow into (C3H × 101)F_1 female recipients who had received 950 r the mean percentage of cells of donor type was estimated as 35 1 The standard error was 4.4 % the modal class centered at 37.5 % the median was 36.6 % and the individual range, among 36 animals tested in two sets of 18 each was 7 to 69 %

Unless otherwise indicated donors and recipients were 12 weeks old at the time of irradiation and bone marrow injection. The conditions for irradiation have been specified elsewhere, as have the procedures for obtaining preparing and injecting the tissues under study[23]

Adult bone marrow, (L × A)F_1 into (C3H × 101)F_1

Figure 1 illustrates the time course of repopulation among the circulating erythrocytes of (C3H × 101)F_1 recipients given 950 r and intravenous injection of 64×10^6 nucleated bone marrow cells from (L × A)F_1 male donors. These data exemplify a rather large number of studies of this combination the figures given are from two separate experiments,

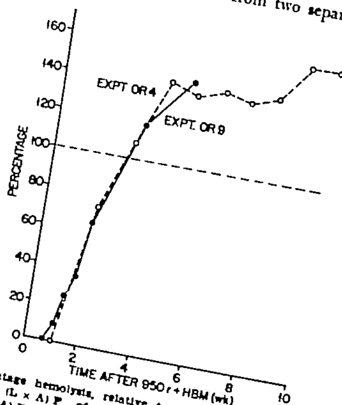


Fig 1 — Percentage hemolysis, relative to normal (L × A) F_1 controls with antiserum specific for (L × A) F_1 of cell suspensions from (C3H × 101) F_1 recipients given 950 r and (L × A) F_1 bone marrow on day O

one involving 60 recipients and the other 40. Each point represents an average of the determinations on 8 mice sampled at each time indicated. Different mice were sampled for successive points. In the first experiment samples were taken at weekly intervals in the second at more frequent intervals during the early part of the response. Several points of interest will be mentioned briefly

(a) Cells of donor specificity can be recognized on the seventh or eighth day after injection of adult homologous marrow into irradiated recipient mice.

(b) These cells of donor origin rapidly increase in number and by about the eighteenth day the total red cell population behaves in the lytic system as though it were 100 % donor cells

(c) The cell population however continues to show increased hemolysis and it does not level off in this respect until sometime between the third and fourth week. The values for excessive hemolysis reflect the greater susceptibility of the total red cell population to specific hemolysis than is characteristic of the red cell population of normal donors who have a sizable fraction of « nonlysable » red cells with most of our test reagents. In the repopulating recipients this fraction of nonlysable cells *is considerably smaller* Simply observing the time course of lysis in a test gave clear indication of the excessive lysability of the repopulating red cells chimeras with a large proportion of positive cells show quick hemolysis, whereas the lysis in suspensions from normal donors is relatively slow Such excessive lysability is not found in the repopulating red cell population of irradiated animals injected with isologous marrow

(d) In this combination the surviving recipients reach a relatively stable level *of excessively lysable cells by about the twenty-sixth day* they continue to display this phenomenon throughout their lives Regression has been observed in other combinations of donor and recipient strains

Repopulation from embryonic and neonatal erythropoietic tissues

Figure shows the time course of erythrocyte repopulation in 40 male (C3H × 101)F_1 12 week-old recipients subjected to 950 r and injected with 35 × 10^6 nucleated cells derived from the livers of 9 newborn and 22 fetal (16-20 day) mice of the inbred strain C57B/10D2 This experiment *was set up by Mrs. Irene Urso. Figure 3 records* the cumulative percentage mortality of the recipient mice together with changes in average body weight for « long term survivors » as compared to averages for the entire experimental population Values for five mice were averaged for each point in Figure 2 except the final one at 130 days which is based on only two as for the data previously presented in this

paper different mice were bled for successive points Two of the mice failed to show a transplant they are not included in the averages Several points of interest may be mentioned

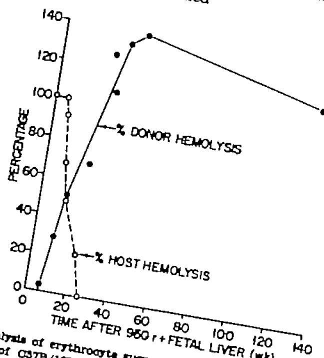


FIG. 2. — Hemolysis of erythrocyte suspensions from (C3H × 101) F_1 mice injected with a suspension of C57B/10D2 fetal and newborn liver cells after 950 r

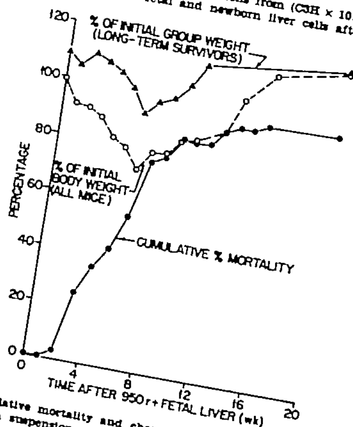


FIG. 3 — Cumulative mortality and changes in body weight of (C3H × 101) F_1 mice injected with a suspension of C57B/10D2 fetal and newborn liver cells after 950 r

(a) The repopulation curve as determined with a donor-specific reagent is very similar to that found for adult bone marrow

(b) Tests with the reagent for host cells reveal that the calculated percentage of host cells falls at a rate compatible with the independent calculations of repopulation by donor cells, and reaches 0 by ~6 days The host cells calculation, however required a correction for a small amount of hemolysis of donor cells in the test reagent which was not completely specific. An indication that the curve for host cells disappearance may be somewhat misleading here was the persistence of agglutination reactions in tests for host cells beyond the time when lytic tests indicated that such cells were no longer present. In view of the generally consistent indications that the host cells have been completely replaced by donor cells by about the twenty-sixth day however the agglutination observed more probably points to an increased agglutinability of donor cells by the cross-reactive antibody for host cells in the reagent Excessive agglutinability as well as lysability is characteristic of the cells of donor origin in these chimeras.

(c) In recipients of this strain combination fetal or neonatal donor tissue seems to have no viability advantage over adult marrow In certain strain combinations, but not in all fetal tissues do appear preferable [3, 8, 24, 33] We have also found that the red cell populations in such combinations are regularly constituted of cells of the normal donor type [23]

(d) The weight record of the few « long term survivors » in this experiment shows that these animals did not escape a reaction but only survived it During the sixth week they entered a period of recovery after which host and transplant appeared to be living together compatibly

(e) The « long-term survivors » were characterized by a higher initial weight than the experimental population as a whole they recovered their initial weight by the second week after irradiation and liver-cell treatment while the general population did not In our experience these two characteristics have frequently but not unfailingly enabled us to pick out the potential long term survivors early in an experiment

OTHER STRAIN COMBINATIONS

Working in a laboratory in which several groups and numerous individuals were investigating various aspects of bone marrow transplantation, I had access to a variety of animals for definition of the status of their erythropoietic transplants Without discussing these studies in detail I should like to submit the following general observations

(a) About 500 (C3H × 101)F_1 recipients given 950 r and (L × A)F_1 bone marrow showed without exception complete and stable repopulation by donor erythrocytes. Several other strain combinations in which the recipients required lower radiation doses to accomplish an effect on viability in untreated animals comparable to that observed with 950 r for (C3H × 101) F_1 showed much less regular repopulation by cells of the donor sort after these lower radiation doses. We cannot assume therefore, that giving a lethal radiation dose automatically ensures a persistent transplant of bone marrow quantitative identification of the transplant is necessary for confident evaluation of the sequellae in each combination.

(b) We cannot assume that because lethally irradiated animals injected with homologous bone marrow survive longer than uninjected irradiated animals of the same strain, those dying later all carry an established transplant. In certain combinations, involving lethal irradiation of animals that had been given no prior immunization the transplant was established only transiently and the only survivors early regressed to host type red cells. Apparently the injected marrow permitted hosts that would ordinarily have died from the irradiation to survive until their own erythropoietic machinery recovered. Whether such animals may in fact be maintaining transplants of other than erythropoietic cells remains to be determined it is entirely possible that establishment of an erythropoietic transplant is not the critical variable in their survival. « Delayed » deaths of animals in such strain combinations and at LD_{100} radiation levels may well depend on an incompletely abrogated host-antigraft immune mechanism. There is no radiation dose level on either an absolute or a relative viability basis that can separate in all strains an early death pattern deriving from host versus-graft reactions from a delayed death pattern deriving from graft versus-host reactions.

(c) In particular combinations persistent transplants have been observed in mice after radiation doses as low as 400 r. A great diversity in the career of transplants has been identified by the application of the erythrocyte marker techniques for example, strain 101 inbred mice receiving 850 r and C57BL bone marrow frequently reverted to host type, and complete reversion among the survivors was observed as early as 43 days after irradiation and bone marrow treatment. Some C57BL × 101 hybrids receiving C57BL marrow after 900 r showed mixtures of recipient and donor type cells when tested at 93 days after the treatment. When spleen cells were injected after 400 r in this combination, some of the animals had erythrocytes entirely of the donor type at the ninety third day some showed all recipient cells and some showed mixtures.

(d) $(L \times A)F_1$ marrow injected into C3H × 101 hybrids after 950 r unfailingly showed a complete repopulation by donor cells, but the admixture of isologous marrow in the injection regularly resulted in the ultimate establishment of cells exclusively of the isologous type. Even at these high radiation doses, therefore, the host cannot be regarded as providing a neutral environment upon which the graft may work its will some elements of the recognition-and response machinery must persist to give a consistent competitive advantage to compatible tissues.

Additional treatments of donor and host

Among other approaches to the bone marrow transplantation system to which the identification of red cells in mice has contributed points of interest a few may be singled out for brief mention here

Preimmunisation of the recipients with fractions of donor tissue confers a relative radioresistance on the immune response machinery of the host. This procedure has been applied particularly by Makinodan and his colleagues to the identification of antigens that may be involved in host versus-graft incompatibility reactions in rat-into-mouse combinations [12] In homologous preimmunization experiments, it soon became evident that preimmunization did not prevent the establishment of a transplant at least of erythropoietic tissues red cells of donor origin appeared in the treated recipients even though a wave of early mortality engulfed most of the experimental population Survivors continued to show normal transplants.

In another set of experiments, animals were preimmunized with pure red cell preparations to test whether the effect of cells presumably lacking in « transplantation antigens » might be to divert the immune responses of the host toward « enhancement » of the transplant Control animals were injected with isologous red cells. The quantitative behavior of the homologous transplant as well as the viability of the hosts, their body weight changes, and all other variables identified were indistinguishable in the experimental and control groups It is not surprising of course that we did not achieve « enhancement » by these procedures the definition of conditions necessary for enhancement in this system may be difficult Note however that no preimmunization death pattern followed this treatment

Some attempts were made to confer tolerance on prospective donors and hosts by injecting bone marrow intracranially into newborn mice Newborn DBA 2 mice were injected with marrow from C57Bl male donors (8 × 10^6 nucleated cells per injection). When these mice were given skin transplants from C57BL donors seven weeks later they showed no clear evidence of tolerance In another experiment bone marrow

suspensions from $(L \times A)F_1$ adult males were injected intracranially (11.8×10^8 nucleated cells per injection) into newborn $(C3H \times 101)F_1$ recipients. These mice were then used when they were twelve weeks old as recipients and donors in reciprocal bone marrow experiments with $(L \times A)F_1$ mice. No sign of tolerance in either host or graft was evident in the delayed death patterns after this experiment. On the contrary the times and frequencies of death suggested some « preimmunizing » effect deriving from the treatment of the neonatal animals. This effect was observed when either the hosts or the donors had received the neonatal injection

General conclusions

This paper was designed to raise points for discussion In general the application of serological marker techniques to bone marrow transplantation experiments contributes information and poses problems of value for reaching an ultimate understanding of this system. The disappearance rate of host cells already circulating at the time of radiation bone marrow treatment seems high as judged by current estimates of the normal life span of erythrocytes in the mouse [22] The possibility that some kind of dynamic interaction of host and graft contributes to this rapid decline of host cells in homologous bone marrow experiments is currently under investigation by Drs. Joan W Goodman and L. H Smith at the Oak Ridge National Laboratory

The irregular experience with erythropoietic tissues of fetal origin among different line combinations is provocative. I should like to suggest the possibility that fetal donor tissues provoke less delayed death than do adult tissues in combinations in which the graft versus-host aspect of delayed incompatibility reactions is the predominant component whereas combinations in which fetal tissues provoke high levels of delayed death may sometimes reflect an important host versus-graft component in the delayed reaction In the experiment with fetal tissue reported here, embryonic or neonatal liver cells gave rise to red cells that were differentiated entirely normally for their H-2 antigens. In no fetal-into-adult combination so far tested has a failure or deviation of normal antigenic development played a discernible part in the « toleration » of the transplant No evidence for « graft adaptation » has been obtained in any of these studies

The choice of strains of mice used in radiation-bone marrow experiments may profoundly affect the kinds of conclusions drawn The time at which death occurs is not in itself an adequate criterion for the causes of death. The true bases of delayed death still escape us there seems to be adequate reason for believing that it rests ultimately on an

incompatibility between host and graft and both *directions of incom*patibility may well be important in the general situation. The « delayed death » phenomenon may reflect quite indirect consequences of an immunological struggle between host and graft leading for example to the depletion of nonspecific components of the immune system of critical importance in the control of endemic pathogens.

This system of transplantation offers valuable tools for fundamental studies of tissue transplantation reactions in themselves, and constantly beckons us into more immediate areas of application. We are still however all too ignorant of the components of this system we cannot generalize even from one mouse strain combination to another and we will *need to know a great deal more before we can feel confident in extend*ing this system to a context of direct utility to man.

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DISCUSSION

MEDAWAR There are three questions I should like to ask Dr Owe (a) Although it would not be suitable for routine analysis, could one no get a very accurate measure of the degree of red cell intermixture by using a fluorescent antibody on fixed blood smears ? (b) In radiation chimeras the donor red cells must all be young that is, recently formed I wasn t clear why Dr Owen said that this could not account for their high lysability (c) Homologous fetal liver cells do not always give the *permanent* cure of radiation injury which one would expect if the « graft versus-host » interpretation of homologous disease were the whole truth. Could this be because the H 2 antigens in fetal liver are not yet fully expressed so that the host does not become tolerant of them ? If the host did not become tolerant one might get a host against-graft reaction when the H 2 antigens eventually became mature.

OWEN (a) We have not been able to differentiate red cell types in mixtures by using fluorescent antibody we suspect that there are too few receptor sites on the cell surface, for the antigens in which we have been interested to fix sufficient antibody for visualization. No doubt this

incompatibility between host and graft and both directions of incompatibility may well be important in the general situation. The « delayed death » phenomenon may reflect quite indirect consequences of an immunological struggle between host and graft leading for example to the depletion of nonspecific components of the immune system of critical importance in the control of endemic pathogens.

This system of transplantation offers valuable tools for fundamental studies of tissue transplantation reactions in themselves, and constantly beckons us into more immediate areas of application We are still however all too ignorant of the components of this system we cannot generalize even from one mouse strain combination to another and we will need to know a great deal more before we can feel confident in extending this system to a context of direct utility to man.

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the host erythrocytes to disappear completely This difference between the homologous and the heterologous chimaeras is most probably not a real one since the electrophoretic method of haemoglobin differentiation is not sensitive enough to detect the presence of less than 10 % of one component in a mixture of CBA and C57BL haemoglobins

Is it possible for Dr Owen to compare his results with those of Makinodan in rat to mouse marrow transplantation where the same recipient strains may have been employed ?

OWEN Yes I believe Makinodan s and my recipients were the same (C3H × 101) F_1 I recognise the difference in the kinetic findings you have just reported and ours. As I indicated in my paper our lytic procedure does not take account of the non lysable fraction of test cells and it is conceivable that host cells persist in that fraction of the cell population for periods that would bring our data into agreement with yours I would have doubted it however because several procedures, including preliminary determinations of the life span of chromium labelled isologous red cells injected into these chimaeras by Drs Joan W Goodman and L. Smith my own serological studies of host-cell disappearance consistent with donor-cell repopulation rates, and the consistency of the serological estimates with haemoglobin determinations by Popp Cosgrove and myself published in *Proc Soc Exp Biol & Med* December 1958 all seem compatible with the observations reported here. It will be very interesting to find whether our differences arise from an artefact if not, something of real significance may await us.

difficulty could be evaded, for example by «layering» techniques or by some such method as mixed agglutination with fluorescent marker cells. I suspect however that an application of tritium-labelled antibody by procedures being developed by Atwood, would offer greater promise. (b) High lysability is not found in red cells deriving from isologous transplants when they are tested with reagents reactive with them (This observation was originally made by Dr Joan W Goodman). It must therefore, be something in their foreign environment that makes the cell population derived from homologous transplants excessively lysable; such non-specific variables as average cell age etc., should not be very different for the two types of transplants. (c) Our studies with red cells repopulating from homologous fetal liver transplants indicate that these cells can be recognised after the same interval and show the same kinds and amounts of H 2 specificities as do cells deriving from adult bone marrow in the same combinations. I would doubt therefore that your suggestion is supportable at least in terms of the serology of H 2 antigens. It is of course possible that other antigens or unrecognized components of H 2 may be found to fit your interesting suggestion in bone marrow as well as fetal liver transplantation.

TRENTIN With regard to the possibility that failure of mice protected with homologous fetal liver to escape delayed mortality in certain strain combinations may be related to a host-versus-graft etiology of delayed mortality in these combinations, I would like to indicate that I have obtained such results in strain combinations and under conditions of irradiation in which a graft-versus-host etiology of delayed mortality seems unlikely. Actually such differences are not unlike the known differences depending on strain combinations in the ability to induce tolerance by injection of homologous spleen into fetal or newborn mice. It is also of interest that most of the cases where better results have been obtained with fetal liver as opposed to adult marrow appear to have involved the C57 strain as donor.

OWEN Thank you. I agree.

VAN BEKKUM There seems indeed to exist a difference between the results obtained by Dr Owen and those we got by using a method of differentiating between host and donor cells (CBA C57Bl combinations) by electrophoresis of the haemoglobins (*Nature* 181 946, 1958). According to our data the replacement of host erythrocytes by donor type erythrocytes seems to take between 45 and 50 days following the irradiation and transplantation. This is somewhat shorter than that observed in rat bone marrow treated mice where it takes between 60 and 70 days for

the host erythrocytes to disappear completely This difference between the homologous and the heterologous chimaeras is most probably not a real one, since the electrophoretic method of haemoglobin differentiation is not sensitive enough to detect the presence of less than 10 % of one component in a mixture of CBA and C57BL haemoglobins

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Grafting rat skin to mouse/rat chimaeras

D W H Barnes, C. E. Ford S. M Gray and J F Loutit

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Congdon and Lorenz [8] demonstrated that mice given lethal doses of X radiation may recover following the administration of rat bone marrow The observation has been repeatedly confirmed and it is now known that the recovery is in virtue of recolonisation of the mouse's damaged haemopoietic tissues with the donated rat-cells (Ford et al. [9] Nowell et al [15] Vos et al [16] Makinodan [13])

Brocades Zaalberg et al [4] have reported that these recovered animals will take grafts of skin from the same strain of rat and in a discussion of a paper by Makinodan [11] at the annual symposium of the Biology Division Oak Ridge we reported verbally similar early success. The results presented now are an extension of that work

The published material from the Netherlands indicated that the acceptance or rejection of the skin-graft depended on the persistence or non-persistence of rat cells as a population in haemopoietic tissues. (The scoring depended on the identification of rat granulocytes in the circulating blood by the histochemical test for alkaline phosphatase). It suggested that the dose of radiation given to induce the chimaerical state was an important factor And it gave the impression that a number of successful « takes » later underwent a « deterioration » Our conclusions are with slight reservations the same as theirs.

Rats The albino rats were drawn from our colony which is believed to stem from Wistar stock. They also have been maintained by strict sib-mating for at least 10 years

X-irradiation The mice were irradiated with X-rays (240 Kv c.p 15 ma HVL 1.2 mm. Cu) at 43 rads/min by the standard technique of the laboratory (Corp [1]).

Suspensions for intravenous therapy Two femurs of a young rat were removed surgically the epiphyses avulsed and the marrow blown out from the shaft with compressed air into 2 ml of 0.9 % saline. The suspension was shaken to disperse the cells drawn up through a size 14 (British Hypodermic) needle into a syringe The syringe was then refitted with a size 18 needle for injection of 0.4 ml intravenously into tail veins of each of 5 mice. This is usually the equivalent of approximately 2×10^7 nucleated cells per mouse.

Skin grafting The method used was a modification of that due to Billingham and Medawar 1951 and has been described elsewhere (Barnes and Loutit [2])

Cytological examination The details of the method have been given previously (Ford and Hamerton [6]) Bone marrow was derived from the two femurs of each animal as a suspension Portions of spleen and thymus and axillary brachial or more usually inguinal lymph nodes were also taken

Results.

Eighteen experiments have been attempted — A to R Table I. The dose of X rays given to the mice varied from 850 to 1200 rads. In all experiments except G the mice, having received the irradiation were injected intravenously with rat bone marrow

In experiment G no bone marrow was given the mice had received 850 rads of X rays, a dose some 25 rads greater than the average LD_{50} and it was hoped to use the possible survivors as controls to mice given the same dose and treated with rat bone marrow Whilst 11 out of 20 survived for 15 days and were then skin-grafted with rat skin none survived sufficiently long thereafter for any useful results to be recordable.

In experiments B and P none of the bone marrow-treated irradiated mice survived beyond two weeks after irradiation and were thus not grafted with skin

In experiments C and D although there were survivors beyond [illegible] weeks and these were skin-grafted they did not survive long enough to give useful results.

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Stocks and Methods

Mice The mice used in these experiments were the Harwell strain of CBA (CBAH) — (Carter et al [3]) — *inbred by strict sib-mating with frequent* re-selection of lines

In the remaining 13 experiments some irradiated, treated and grafted mice did survive for at least 1 month after the skin-grafting and the results in 79 individuals are presented in Table II.

Clearly mice given 950 rads or more and restored immediately with rat bone marrow will usually take skin-grafts from rats, applied 2 weeks or more after the irradiation. All 7 out of 7 mice given more than

TABLE II

Number of mice with surviving grafts of rat skin as a fraction of the total number grafted one month earlier

Experiment No.	Dose of X-rays given in rads					
	850	900	950	1050	1100	1200
A	5/5	—	—	—	—	—
E	0/8	0/4	—	—	—	—
F	—	—	1/2	—	—	—
H	5/7	—	—	—	—	—
I	—	—	10/11	—	—	—
J	0/5	—	—	—	—	—
K	2/2	—	5/7	2/2	—	—
L	—	—	5/5	—	—	—
M	—	—	—	2/2	—	—
N	—	—	3/3	—	—	—
O	—	—	10/11	—	—	—
Q	—	—	—	—	2/2	1/1
R	—	—	1/1	—	—	—
Total	18/28	0/4	35/40	4/4	2/2	1/1

TABLE III

Number of mice with surviving grafts of rat skin as a fraction of the total number grafted 2 months earlier

Experiment No.	Dose of X-rays given in rads			
	850	900	950	1050
A	4/4	—	—	—
E	0/7	0/3	—	—
F	—	—	0/2	—
H	5/7	—	—	—
I	—	—	9/9	—
J	0/2	—	—	—
K	1/2	—	3/4	1/1
L, M, N, O, Q, R	No surviving mice			
Total	10/24	0/3	12/15	1/1

Over half soon died or were sacrificed when in poor general condition but with their skin-grafts still intact. Of the remaining 11 which were in good clinical state and with healthy rat skin grafts, 8 were used for a consequential experiment and 3 were maintained without further interference. These 3 all lived for a year or more and were then sacrificed. (d) of experiment A and (h) of experiment I both had good healthy rat skin at sacrifice (i) of experiment I had retained its rat skin for more than 6 months when the graft slowly atrophied.

Cytological examination of the chromosomes of cells of the myeloid and lymphatic tissues of the 9 animals sacrificed showed that the cells in mitosis were either wholly of rat origin (3 cases) wholly of mouse origin (2 cases) or mixed (4 cases) The details are given in the Appendix

TABLE V

Cytological findings in myeloid and lymphatic tissues in animals sacrificed within 10 weeks of irradiation or 2 months of skin-grafting

Expt. No	*X-ray dose rads*	*Time in days X-ray to graft + graft to sacrifice*	*Condition of graft*	*Cytology of cells in mitosis in haemopoietic tissue M = Mouse R = Rat*	*Ref No in appendix.*
A	850	14 + 9	? septic	M > R	858
		14 + 34	Intact	R	938
B	850	12 —	—	R	866
		14 —	—	M	878
H	850	15 + 12	Healing	M > R	1230
K	950	14 + 12	Healing	M ≃ R	1387
	1050	14 + 14	»	R	1391
		14 + 28	Intact	R	1402
L	950	14 + 35	Intact	M > R	1421
N	950	15 + 34	Intact	M ≃ R	1516
		15 + 41	»	M > R	1534
		15 + 41	»	M > R	1535
O	950	15 + 32	Intact	R > M	1517
		15 + 32	»	R — M	1518
		15 + 49	»	R	1552
		15 + 49	»	R ≫ M	1553
		15 + 49	»	R ≃ M	1554
		15 + 53	»	R ≫ M	1556
		15 + 53	»	R ≫ M	1559
		15 + 54	»	R ≫ M	1560
		15 + 54	»	R	1561
Q	1100	13 + 30	Intact	R ≫ M	1593

( ) Animals not grafted — = time in days X-ray to sacrifice

1000 rads accepted the skin and 35 out of 40 mice given 950 rads bore living rat skin after a month However, following the smaller doses 850 and 900 rads, the results were more variable None of the 4 mice given 900 rads accepted the rat skin though 13 of the larger group of 28 given 850 rads did so

The mice available for scoring at 2 months after grafting are very much fewer in number Death from the so-called « secondary disease » will have accounted for many of the animals even within one month of the grafting and in the next months deaths from this cause continue Table III gives the score for the 43 animals still alive after 60 days.

This pattern is the same as in Table II but there are relatively far fewer animals having received the higher doses of X rays.

The fate of the 23 animals with skin-grafts intact after 2 months is given in Table IV

TABLE IV

Fate of 23 mice bearing grafts of rat skin for at least 2 months

Expt. No	X-ray dose received rads	Fate of animal	Cytology of cells in mitosis in myeloid and lymphatic tissues M = Mouse R = Rat	Ref No Appendix.
A	850	(a) S — 62 ( )	M > R	1025
		(b) D — 70	—	—
		(c) S — 136	M	1191
		(e) S — 355	R > M	1839
H	850	(a) S — 60	M	1327
		(b) S — 60	M + R	1330
		(c) S — 76	R	1375
		(d) D — 96		—
		(e) Intact 100 ( )		—
I	950	(a) D — 80	—	—
		(b)		—
		(c) Intact 84 ( )	—	—
		(d)		—
		(e)		—
		(f) D — 92	—	
		(g) S 169	R	1443
		(h) S 440	R	1871
		(i) S 440	R M	1872
K	850	(a) D 62		
	950	(a) D 63		
		(b) Intact 80 ( )		
		(c)		
	1050	(a) Intact 80 ( )		

( ) S sacrified D died numeral number of d y after skin-gr ft g

( ) Used for consequential experiment

mitosis in the haemopoietic tissues. In one of those three (exp. F) a third skin graft applied 4 days before sacrifice was on histological examination vascularised and showed continuity of mouse and rat epithelia.

Discussion.

The identification of origin of cell populations in haemopoietic tissues.

The cytological identification of dividing rat cells in the bone marrow, spleen and lymphatic tissues of heterologous chimaeras of mouse and rat has been reported previously from this laboratory (Ford et al. [9, 11]) and from Oak Ridge by Gengozian et al. [13]. Some features of the reversal with time of the cell-populations from rat-type to mouse type were discussed by us later (Barnes et al. [1]). The cytological data given in the section on *results* are an amplification and extension of our previous reports.

The results set out in detail in the Appendix may give the impression that scoring was done in an erratic manner. In fact the great variation in numbers of mitotic cells recorded was determined largely by the frequency of mitosis in the different tissues examined and the technical standard attained. As a rule two preparations were examined from each tissue and it may have taken as long to find and score 3 cells in poor material with few mitoses as 300 in technically good preparations where mitoses were abundant. An attempt was made to classify all the cells seen in metaphase but some 2 % had to be rejected as unclassifiable because of poor fixation or clumping of the chromosomes.

An important point which emerges from examination of the full cytological data is that, in general, there is a remarkably good correlation between the tissues of a single animal in respect of the proportion of dividing rat and mouse cells observed. Mouse 1230 is the most striking exception to this rule. Here the disparity is so great (all rat cells in the bone marrow, all mouse cells in the other 3 tissues) that it is reasonable to suspect an error either to recording or labelling. However a careful check at the time the original observations were made and a later recheck make error of either kind very unlikely. Subsequently another mouse (1387) was found to be very similar (almost all rat cells in the bone marrow, only mouse cells in the spleen). Mouse 1516 is a third example in which there is a considerable variation between different tissues.

Another point worthy of notice is that in most of the animals sacrificed for cytological examination the dividing cells were either wholly or very largely rat (17) or wholly or very largely mouse (20): there were relatively few intermediates (8) (Table VII).

Previous experience indicates that by 50 days after irradiation (950 rads) and treatment the mouse/rat chimaera normally has a fully

mitosis in the haemopoietic tissues. In one of those three (exp. F) a third skin graft applied 4 days before sacrifice was on histological examination vascularised and showed continuity of mouse and rat epithelia

Discussion

The identification of origin of cell populations in haemopoietic tissues

The cytological identification of dividing rat cells in the bone marrow spleen and lymphatic tissues of heterologous chimaeras of mouse and rat has been reported previously from this laboratory (Ford et al [10, 11]) and from Oak Ridge by Gengozian et al [12] Some features of the reversal with time of the cell-populations from rat type to mouse type were discussed by us later (Barnes et al. [1]) The cytological data given in the section on *results* are an amplification and extension of our previous reports.

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Another point worthy of notice is that in most of the animals sacrificed for cytological examination the dividing cells were either wholly or very largely rat (17) or wholly or very largely mouse (20) there were relatively few intermediates (8) (Table VII).

Previous experience indicates that by 30 days after irradiation (950 rads) and treatment the mouse/rat chimaera normally has a fully

established graft of rat cells and that few or no dividing mouse cells are present (Ford et al [11]) However exceptions to this rule do occur more particularly in the lymphatic tissue (Ford et al [14]).

TABLE VII.

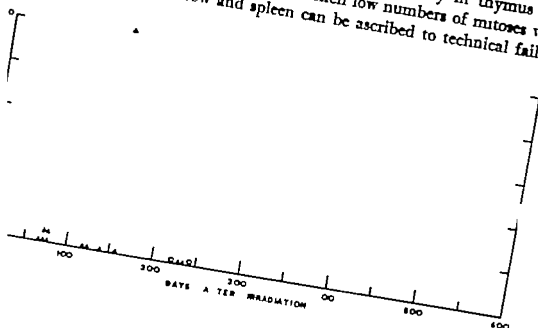
Mice sacrificed for cytological examination classified according to the proportion of mouse cells to total in mitosis.

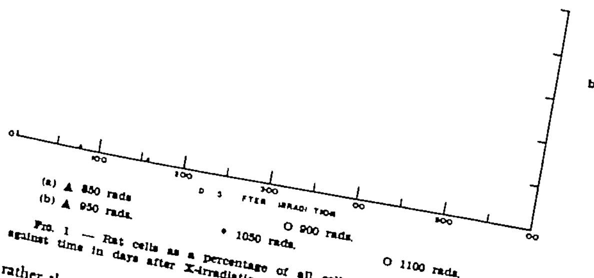
Percent mouse cells.	Number of animals and dose in rads					
	850	900	950	1050	1100	Total
0	4		4	2		10
0 — 5	1		5		1	7
5 — 10	0		0			0
10 — 90	2		6			8
90 — 95	0		1			1
95 — 100	3		2			5
100	10	2	[illegible]			14

In the present series of tests we cannot be sure that rat cells became the dominant component of the partnership at an early stage in all the mice, although our experience with « low dose chimaeras (CBA hosts 575 rads T6 donors (Ford et al ') CBA hosts, 700 rads rat donors *) would make it probable. If they did the low proportion of chimaeras with substantial numbers of cells of each type shows that reversion to host tissue must have taken place very rapidly once the process began particularly in the 850 rads series The 3 examples quoted above of non conformity with the rule that similar proportions of rat and mouse cells are normally present in each of the different tissues of a chimaera may now be interpreted as animals in this phase of rapid transition of the haemopoietic cell populations from graft back to host In any case the results strongly suggest that if the chimaera survives long enough the proportion of rat to mouse cells in the mixture ultimately attains one of two stable states either predominantly rat or predominantly mouse Our earlier conclusion (Ford et al. [11]) that in mouse/rat chimaeras established after an X-ray dose of 950 rads « the grafts are ultimately although perhaps never quite completely replaced by cells of the host is now seen to be incomplete These points are illustrated by Figs 1 a and b in which the data of the Appendix are presented in graphical form each point referring to a single animal and representing the percentage of rat cells in the total of mitotic cells recorded summed over all tissues.

( ) Unpublished information

A final point to which attention should be drawn is the marked variation between different animals in mitotic activity in thymus and lymph nodes. (The few instances in which low numbers of mitoses were recorded in bone marrow and spleen can be ascribed to technical failure





(a) ▲ 850 rads. (b) ▲ 950 rads. O 900 rads. • 1050 rads. O 1100 rads.

FIG. 1 — Rat cells as a percentage of all cells observed in mitosis plotted against time in days after X-irradiation. Each point represents a single animal

rather than an inherently low frequency of cellular proliferation) It is commonly found that the lymphatic tissues of homologous and heterologous chimaeras never really recover after exposure to irradiation but become reduced in size and fibrotic. In Feulgen-squash preparations such tissues appear as clumps and groups of capsular or stromal cells with

few free cells and none or virtually none in mitosis. That fibrosis is by no means universal is demonstrated by the high level of mitotic activity in the thymus of mouse 1434 and in lymph node of mouse 1025 230 and 76 days after irradiation respectively. In these two examples the haemopoietic tissues had wholly reverted to mouse, although this is not a necessary condition for high mitotic activity as shown in both thymus and lymph node of mouse 1514 in which many rat cells (but only rat cells) were found in mitosis, 137 days after irradiation. No explanation can yet be offered for this great variation in behaviour of lymphatic tissue.

Acceptance and rejection of rat skin-grafts

The average heterologous mouse/rat chimaera certainly in our hands is not a thrifty animal. Most of our experience is derived from experiments where CBAH mice have been irradiated with 950 rads of X rays and then treated with rat bone marrow. The percentage cumulative mortality rises approximately linearly with long time.

A similar graph for homologous chimaeras on the other hand is usually characterized by waves presumably indicative of specific causes of death at the respective times (Barnes — to be published).

It would seem that for the heterologous chimaera some non-specific factor is more potent than specific causes. In this series of experiments the dose of X rays was not kept constant at 950 rads but was varied from 850 to 1200 rads. There were however substantial numbers of animals only in the groups given 850 rads (LD_{44}) and 950 rads (LD_{11}) and after two weeks or so further stress was given in the form of skin-grafting. Nevertheless selecting from Table I the groups where mortality is reported at 2 3 weeks [i.e. omitting experiments D F (part) F and I where skin grafting was delayed and experiment G where no rat bone marrow was given] one can derive the surviving fractions at 2 3 weeks for the bone-marrow treated animals. These are 48/85 for 850 rads 9 15 for 900 rads, 48/86 for 950 rads 4/10 for 1000 rads, 14/30 for 1050 rads 2/20 for 1100 rads 0/20 for 1150 rads and 2 20 for 1200 rads.

In fact the mortality is roughly 50 % at 2 3 weeks for each dose of radiation up to 1050 rads. At these levels therefore there seems to be no great difference in non-specific forces.

Grafts of rat skin applied to these non thrifty heterologous mouse/rat chimaeras in practically all cases heal in and start growing hair provided the dose of X rays given has been 950 rads or more (Table II).

(a) Chimaeras following doses of X rays less than 950 rads.

Following doses of X rays less than 950 rads the results were variable in some experiments the grafts took well initially in others they were

rejected in less than a month If there is a threshold of absorbed dose necessary to abolish completely the host mouse's normal immunological defences which is a requisite for the « take » of a foreign graft, it could be that there was a variation between experiments in the absorbed doses due to random variation in the way the mice were distributed in their boxes there should be only 3 % variation in the doses of X rays applied. It could also be that there was variation in sensitivity in the stock despite close in breeding If the rat skin is rejected because the host's immunological reaction has not been entirely abolished, the rat cells injected earlier as suspensions of bone marrow should also have been eliminated Only one animal (Experiment A — Table V) was sacrificed early with a graft sloughing — and this may have been due to sepsis — and its haemopoietic tissue did show some rat cells in mitosis so that in all probability the loss of this skin graft was due to technical faults. However numerous animals were sacrificed considerably later when they had rejected 2 or more successive skin grafts and in these no rat cells in mitosis were identified The most probable explanation in these 9 instances (Experiments E.H.J Table VI) is that with time there had been a complete reversion of the cell populations to mouse and late recovery of muring immunological specificity causing elimination of all rat cells

There were in addition the two instances (Experiment K, Table VI) where the primary skin graft atrophied late after an apparent « take » at 1 month. These mice, on sacrifice after 4 months, showed abundant rat cells in mitosis in haemopoietic tissues presumably therefore the loss of the rat skin-graft was due to some local physiological inadequacy not immunological recovery of the host. Finally some skin grafts (10/24, Table III) did persist for two months one as long as a year and 6 of these animals were sacrificed. In 4 of them rat cells were demonstrable but the other 2 revealed only mouse cells. The failure to find rat cells in mitosis does not prove absence of rat cells. They may be merely dormant overgrown by mouse cells physiologically more competent in the environment and immunologically tolerant of rat-cells since they have matured in the presence of rat tissue (Barnes et al [1]) in fact one of the other 4 had only a single rat cell in mitosis to some 400 mouse cells scored One cannot exclude of course, since the cytological scoring is done at one point in time, that at this time a recovery of the host's immunological reactivity is just being completed and that the skin graft would have been shed shortly thereafter

(b) Chimaeras following a dose of X rays 950 rads and over

When the mice were given 950 rads and over and resuscitated with rat bone marrow rat skin subsequently applied was in practically all cases satisfactorily retained 12 out of the 15 mice given 950 rads and living

for more than 2 months after skin grafting, retained their skin grafts so did the one animal surviving *for more than 2 months* after skin grafting following 1050 rads. In only 3 of these were the marrow and lymph tissues examined cytologically and all had wholly or substantially rat cells in mitosis (Table IV) but of the 17 animals sacrificed earlier 2 to 7 weeks after apparently successful skin grafting all had rat cells either as the sole or as an abundant population of mitotic cells (Table V). Three animals which had lost rat skin grafts (Table VI) were also examined 2 showed *mouse cells only in mitosis.* As *mouse 1426* (Expt. K) had shed its only skin graft and as mouse 1290 (Expt I) had shed 2 skin grafts quickly they probably represent a failure completely to abolish the murine specificity The third mouse 1273 (Expt F) grafted nearly 9 months after its irradiation and recovery following rat marrow had shown a slow atrophy of the skin graft and its haemopoietic cells did include some rat cells (Table VI).

Correlation between results of cytological examination and skin grafting

In general when grafts of rat skin persisted on the mouse/rat heterologous chimaera cytological examination of the actively dividing myeloid *tissues or the usually rather atrophic lymphatic tissues or both* revealed rat-cells in mitosis The few exceptions have to be explained on the hypothesis that rat-cells were in fact present but were a minority population with little active division Where mouse cells were present in the haemopoietic tissues, particularly as the dominant population immunological tolerance of these cells for rat tissues has been postulated (Barnes et al [2]) and it has been concluded that these cells replace the earlier population of rat-cells through their greater physiological competence in the environment (Ford et al. [11] Barnes et al [1]).

When grafts of rat skin were rejected by the animals cytological examination usually revealed only mouse-cells in the haemopoietic tissues. In the majority of these cases repeated rejections of rat skin had been recorded It is postulated that in these cases reversal to murine cells had not resulted in tolerance and that as well as being more physiologically competent the mouse-cells had effected an immunological response against rat tissue In a smaller group loss of the skin graft was slow and rat cells were present on cytological examination of haemopoietic tissues. The loss of skin in these cases therefore did not appear to be due to an immunological reaction and it could be due to some physiological inadequacy

After 950 rads and more there was a greater chance of persistence of the skin-graft and of dividing rat-cells in the haemopoietic tissues. After 850 rads, though there were instances of persistence the commoner

reaction was for rejection presumably due to recovery of the host's immunological mechanisms.

Our data on long term survivors are too limited for the deduction of firm conclusions. The 2 animals (1539, Expt. A and 1871 Expt. I) which lived for a year with intact skin grafts had rat-type haemopoietic tissue, but so did 1 animal (1872 Expt. I) living as long but with its skin graft deteriorating after six months.

Complete persistence of rat haemopoietic tissue may be necessary for the full nutrition of the rat skin but does not necessarily insure it Survival and function for 2 months or more is not incompatible with a mixed population of haemopoietic cells.

SUMMARY

Heterologous chimaeras produced by irradiating CBAH mice with X rays (850 to 1200 rads) and injecting with rat bone marrow have been skin grafted with rat skin. These animals usually live only a few weeks or a few months Provided the X ray dose has been 950 rads or more, with few exceptions they retain both their skin grafts and rat cells identified cytologically in their haemopoietic tissues. At lower doses while retention is not exceptional the majority lose the rat cells from their haemopoietic tissues and their rat skin presumably by recovery of the host's immunological reactivity In the very few long lived heterologous chimaeras loss of a skin graft previously healthy may occur late by a slow atrophic deterioration. This may occur when the haemopoietic tissues are populated partially or solely with rat cells and is presumably not due to immunological but to physiological causes

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1290	950	138	47	0	199	0	219	0	47	0	68
1449	950	230	139	7	0	103	0			1	0
1871	950	531	440	156	0	16	0			7	7
1872	950	531	440	97	1	147	14				
1433	850	124	108	0	136	0	217	0	142	0	13
1446	850	137	111	0	60	0	440				
1445	850	131	115	0	256	0	23				
1387	950	26	13	37	3	0	92	X	X	X	X
1391	1050	38	14	88	0	X	X	X	X	X	X
1402	1050	39	23	231	0	153	0				
1426	950	66	52	0	213						
1514	850	137	123	164	0	30	0	180	0	88	0
1515	850	137	123	48	47	27	207	3	21	14	21
1421	950	49	33			29	183			1	3
1516	950	49	34	61	172	15	104	X	X	20	2
1534	950	56	41	4	22	1	97			0	1
1535	950	56	41	0	103	22	178			1	1
1517	960	47	33	3	1	17	4	X	X	X	X
1518	956	47	33	X	X	157	148	X	X	8	3
1552	950	64	49	147	0	200	0	1	0	X	X
1553	950	64	49	302	0	23	1	X	X	X	X
1554	950	64	49	85	39	96	103	0	0	2	1
1558	950	68	53	106	0	200	0	1	1	18	0
1559	950	68	53	100	3	159	3	32	0	68	1
1560	950	68	54	38	1	197	2	46	0	49	2
1561	950	69	64	X	X	42	0		X	28	0
1593	1100	63	50	50	0	50	1	1	0	4	0

DISCUSSION

WOODRUFF I am disturbed by Dr Loutit's suggestion that the slow atypical rejection of a skin heterograft which he observed in some animals was not immunological in origin because for several years we have regarded this phenomenon as indicative of partial tolerance in homologous systems and if Dr Loutit's interpretation is correct we shall have to revise our ideas. Before doing this it would be helpful to know how a second skin heterograft from the same donor or a sib of the same sex would behave in Loutit's experiment If it were found as I suspect that the skin graft rejection was a manifestation of partial tolerance it would be interesting in the light of the observations I described yesterday in human twin chimeras, to know whether the state of chimerism persisted in Loutit's animals following slow skin graft rejection and if so for how long

One other point can we exclude the possibility that skin grafts which survived were slowly replaced by host cells as appear to have occurred in one of my human twins?

LOUTIT May I take your second point first? I had meant to bring lantern slides to demonstrate the genuine persistence of the rat skin graft residing on the mouse rat chimaera. Unfortunately I forgot this in the rush of departure However to naked-eye examination the skin appears indubitably of rat type The skin has been taken either from the body or from the tail Body-skin which has taken develops after a few weeks the characteristic long coarse hair of the rat and the carriage of such hair is regarded as essential for the scoring of «take» and persistence of the graft Tail skin retains the coarse rasp-like properties of the donor rat and is readily distinguishable from the thin pliable mouse-skin Sections have been taken only at death of the animals Those on animals recorded as «takes» show the thicker epithelium of the rat-skin healed to the naturally thin mouse epithelium

Now to deal with your first question the indolent loss of what was recorded as initially a graft take entails a slow loss of hair and a gradual thinning and slow deterioration of the graft which may take many weeks to complete I am indeed interested to learn that such slow sheddings are in accord with experience of homografts Physiological factors rather than immunological were invoked in our case since the ultimate loss of the skin graft did not entail simultaneous or subsequent loss of the haemopoietic cells of donor origin

COHEN What is the fate of skin graft within your colony of rats?

No comprehensive statistical study has been made but pilot studies led us to believe that our stock was sufficiently homogeneous. Furthermore, in each case, the donor of the rat-skin has been a sibling of the same sex as the rat which provided the bone marrow used to restore the irradiated CBA mouse.

The pathogenesis of the secondary disease following foreign bone marrow transplantation in irradiated mice

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In many mammalian species the acute mortality following total body X irradiation can be prevented by the administration of viable hematopoietic cells shortly after the irradiation (see review article [1]). The majority of the data concerning this therapeutical approach have been obtained with mice. When irradiated mice are treated with isologous bone marrow or spleen cells the results are usually excellent: acute (30 days) mortality can be completely prevented provided the X ray dose remains below the range in which the intestinal damage is the principal cause of death. The required number of viable nucleated cells from *isologous donors is amazingly small namely* 10^{5} *for bone marrow and* 10^{6} for spleen when the injection is made intravenously [2]. The results of follow-up studies extending after the 30th day have been unequivocal in that mortality is negligible in some laboratories and considerable in other. However the effect of the dose of radiation on long term survival following isologous bone marrow treatment has not been investigated adequately, the available information from each laboratory being limited to one X ray dose that approximates the LD for the particular strains employed. In recent experiments we found that a secondary mortality occurs in CBA mice after isologous bone marrow treatment if the X ray dose is increased. Following 675 r (LD$_{50}$) transplantation of isologous bone marrow causes approximately 100 % of our CBA mice to live beyond the 100th day post irradiation. In contrast about 50 % of similarly treated CBA mice died between the 30th and the 100th day following X irradiation with a dose of 950 r (*). No symptoms charac

(*) See table 1

teristic of secondary disease were observed in these animals, although in a few cages loose faeces were found occasionally. This disturbance lasted for a few days only and it never approached the severe form that was seen in groups receiving rat bone marrow and homologous bone marrow. It is of interest that the highest secondary death rate occurred in the *isologous* CBA combination after 950 r between the 30th and the 60th day post irradiation which is exactly the period of secondary death after homologous and heterologous bone marrow treatment. Following treatment with F_1 bone marrow CBA mice showed no secondary mortality following 675 r and 800 r while following 950 r 50 % of the mice died between days 30 and 100 post irradiation.

When foreign bone marrow—either homologous or heterologous—is used for treatment, the requirements as well as the eventual outcome prove to be quite different. The number of hematopoietic cells required to afford an equivalent protection is considerably greater than with isologous cells. With intravenously administered bone marrow cells this difference amounts to a factor of about twenty[3]. In addition foreign bone marrow treatment is effective only after radiation doses that approximate the LD_{100}; after smaller doses the administration of foreign bone marrow may even be harmful[6, 11].

Provided a proper X-ray dose as well as a sufficiently large number of foreign bone marrow cells have been administered a very satisfactory 30 day survival rate up to 100 % and a complete replacement of the hosts hematopoietic system may be obtained. However beginning in the 5th week and in some experiments even a week earlier the animals become ill and the majority dies before the 100th day post irradiation. This *secondary disease* is clearly distinct from the acute radiation syndrome with the peak of mortality during the second week which develops in irradiated mice after lethal doses of radiation. The secondary disease is characterised by three symptoms: emaciation, the passing of large amounts of loose faeces (diarrhea) and dermatitis accompanied by loss of hair and a delay of the radiation induced depigmentation of the fur. Since the characteristic secondary disease has only been observed following the transplantation of homologous or heterologous hematopoietic tissue (it has also been named homologous disease and foreign bone marrow disease) an immunological reaction between the host and the donor tissues has been postulated as the causative factor. Both antibody production by the host against the foreign hematopoietic tissue and by the graft against the host tissues have been proposed by various investigators (the reader is referred to the papers and discussions of the 1957 Gatlinburg Conference[11]). The latter mechanism has been advocated most strongly by Loutit and his collaborators but neither these

authors nor others have been able to provide direct proof of the existence of such a reaction.

We have previously pointed out that although the basic cause of the secondary disease is probably a graft anti-host reaction some of the symptoms and even the ensuing mortality may well be due to a general decrease of the immunological defense reactions of the animals[3]. In addition late radiation damage has been postulated to be involved[3, 4].

In studying the nature of the secondary disease various approaches have been used.

The genetic approach

This method has been employed by Uphoff[11, 12] and Trentin[13] as well as by van Bekkum and Vos[4, 14]. The inability of F_1 hybrids to react against tissues of either of the parent strains is used to exclude the graft anti host reaction or the host anti-graft reaction from the experimental conditions by grafting F_1 hybrid marrow into irradiated parent strain mice and reversely.

In a series of experiments Uphoff studied the occurrence of secondary disease in irradiated F_1 hybrids that received bone marrow transplants from one of the parent strains. A number of inbred strains and their F_1 hybrids was investigated. In every combination except one secondary disease developed while the mice that received isologous (F_1 hybrid) bone marrow remained free of the disease. The one exception was provided by an F_1 hybrid whose parent strains (BALB/c and DBA/2) were both of the H 2d phenotype. The H locus is known to be the most important of the histocompatibility loci.

The results obtained by Uphoff with the reverse combination (parent strain mice treated with F_1 hybrid bone marrow) are strikingly different in showing a complete absence of secondary disease. In a few experiments however some secondary mortality occurred but the incidence was definitely less than in the case of F_1 hybrids treated with parent strain bone marrow. Trentin's report has been rather conflicting because a considerable secondary mortality occurred in some of his experiments with irradiated parent strain mice that were treated with F_1 hybrid bone marrow. Our own results again differed from those of the two previous authors. With CBA C57Bl and F_1 (CBA ♂ × C57Bl ♀) mice none of the various parent F_1 combinations showed an appreciable incidence of secondary disease or mortality.

However the results of subsequent experiments seem to offer an explanation for this discrepancy. By the use of various X-ray doses followed by treatment with isologous or foreign bone marrow it has been possible to distinguish between late radiation effects and effects

which result from an immunological reaction of the bone marrow graft against the host (Table I). It was mentioned before that a considerable

TABLE I. — *Secondary mortality in isologous and F_1 parent strain combinations following supralethal X-ray doses*

Recipient	Donor	X-ray dose in r	Secondary mortality (between 30 and 100 days post-irradiation)
CBA	CBA	675	none
»	»	800	negligible
»	»	950	50 %
CBA	F_1 hybrid	675	none
»	»	800	none
»	»	950	50
F_1 hybrid	F_1 hybrid	700, 750	none
»	»	950	negligible
F_1 hybrid	CBA	700, 750	none
»	»	800	none
»	»	950	slight, but severe diarrhea.

secondary mortality occurred in CBA mice subjected to 950 r followed by treatment with either isologous or F_1 hybrid marrow. In the first combination immunological factors can safely be excluded since the strain is homogeneous according to the results of intrastrain skin grafting. The secondary mortality is therefore ascribed to late radiation damage. In the second case only an immunological reaction of the host directed against the graft is theoretically possible. However the same combination showed no secondary mortality at all following 675 r and 800 r, both doses which would be expected to cause less inhibition of the hosts' immunological reactivity than a dose of 950 r. Here again the most likely explanation seems that the secondary mortality after 950 r is due to late radiation damage of the CBA recipients.

Following treatment with isologous bone marrow F_1 mice showed a negligible secondary mortality and no secondary disease even after 950 r which shows that the F_1 mouse is much less sensitive to delayed radiation damage than the CBA mouse. After treatment with CBA marrow some secondary mortality occurred in the 950 r F_1 group and diarrhea was noted in all cages from the 3rd until the 7th week. In more than 50 % of the cages the diarrhea was quite severe for some time when the condition could not be distinguished from that seen after rat bone marrow treatment. In the 800 r group diarrhea was less frequently observed and of a milder degree. Except in the rat bone

authors nor others have been able to provide direct proof of the existence of such a reaction.

We have previously pointed out that although the basic cause of the secondary disease is probably a graft anti host reaction, some of the symptoms and even the ensuing mortality may well be due to a general decrease of the immunological defense reactions of the animals [1] In addition late radiation damage has been postulated to be involved [1, 4]

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1) sensitivity of F_1 mice to the heterologous immunological reaction is similar to that of CBA mice

2) secondary death in F_1 mice is conditioned by the presence of foreign bone marrow

The relative resistance of F_1 mice to parent strain bone marrow may be due to a relatively smaller antigenic stimulus in this combination as compared to strictly homologous or heterologous combinations.

In this particular combination (F_1 mice treated with parent strain bone marrow) the mortality after 950 r and the diarrhea that developed after 800 r and 950 r seem to be best interpreted as being at least partly the result of an immunological reaction of the graft against the host. The fact that the diarrhea was more severe in the 950 r group than in the 800 r group as well as the occurrence of slight diarrhea in isologous bone marrow treated CBA mice that were subjected to a dose of 950 r shows that radiation damage is also involved in the development of this condition.

THE STUDY OF THE FUNCTIONAL STATE OF THE BONE MARROW TRANSPLANT IN RELATION TO THE DEVELOPMENT OF THE SECONDARY DISEASE.

The use of rat bone marrow to protect irradiated mice has allowed an evaluation of the functional state of the transplanted hematopoietic system at any time by determining the origin of the erythrocytes and the granulocytes [3]. In accordance with others it was found that survival of the irradiated host animals is accompanied by colonization and proliferation of the donor cells. Failure to survive after median lethal doses of radiation was found to be due to a secondary loss of the rat hematopoietic tissue [7]. The latter reaction usually occurs during the second week after the irradiation and will be referred to as the *acute rejection of the graft*. It is apparently caused by an immunological reaction of the host against the grafted tissue; the incidence of acute rejection of the bone marrow graft is dependent on the dose of radiation and probably also on the antigenic difference between the host and the donor [8]. Our results with F_1 hybrid-parent strain combinations are in complete agreement with this viewpoint. Sub-lethally

irradiated parent strain mice show 100 % mortality when treated with homologous bone marrow only 65 % mortality after treatment with F_1 hybrid marrow F_1 hybrid mice are completely protected by parent strain bone marrow at all radiation dosages (unpublished results)

A delayed host anti-graft reaction is presumably acting in the few animals that show a *gradual* return of host type cells with concomitant loss of the foreign (rat) bone marrow after the latter has initially fully repopulated the hosts tissues. Animals that eventually regain their own hematopoietic system completely have been called « total reversals » In our experience the reversing process is nearly always completed before the end of the 3rd month after the transplantation. Exceptionally the reversion remains incomplete for a very long time, during which the mice maintain a mixed population of host and donor type blood cells [4] These animals will be called « partial reversals » while the term true chimeras has been reserved for those mice that show donor type cells only in the peripheral blood.

The above reaction may be classified as a *delayed rejection of the bone marrow graft* The results of skin grafting experiments with these animals indicate a return of the hosts immunological reactivity owards the donors tissue [5]

It has been shown that the incidence of the delayed graft rejection is also dependent on X ray dose if the X ray dose is increased the incidence of this reaction decreases [18]

The incidence of partial and total reversals among the survivals of the first 100 days post irradiation was presented in detail elsewhere [18] Because the data contribute to an understanding of the mechanism of secondary disease, they will be briefly summarized. Out of a group of 702 rat bone marrow treated mice which received a dose of 675 r (LD_{100}) 126 survived the critical period of secondary disease. At the 100th day 74 or 59 % showed a partial or complete recovery of the hosts hematopoiesis. At about 300 days after irradiation only 45 mice were living, 15 of these were true chimeras 8 were partial reversals and 22 were total reversals. In contrast, from a group of 65 mice that received 800 r and rat bone marrow 27 were living after 100 days (2 partial reversals and 25 true chimeras) and after 300 days only 5 mice had survived all of which were true chimeras. Apparently recovery of the hosts blood forming system is less likely to occur if the dose of irradiation is increased It has been our experience that the few mice that survive for a very long period—e g more than 1 1/2 year—following 675 r and rat bone marrow grafting were nearly all found to be total reversals. Total reversals were always free of the skin lesions which we find characteristic of secondary disease. The incidence of these lesions is highest among true chimeras. These observations indicate that the

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presence of a functionally active graft is required for the maintainance of secondary disease.

Attempts to induce secondary disease symptoms and mortality by the transplantation of foreign lymphoid tissue into irradiated and non irradiated recipients and by parabiosis.

In another paper the administration of homologous lymph node cells in addition to isologous or homologous bone marrow was reported to annihilate the beneficial effects of the bone marrow after total body irradiation [14]

Evidence was provided to show that the grafted lymphoid cells react against the host animal Furthermore a disease resembling the secondary or foreign bone marrow disease was induced in F_1 mice after total body irradiation (700 r) followed by the administration of parent strain bone marrow and small numbers of parent strain lymph node cells.

In non irradiated F_1 mice some symptoms closely resembling those found in the secondary disease, were evoked by the injection of lymph node cells obtained from parent strain mice that had been sensitized previously against the tissues of the other parent strain

Finally similar symptoms as well as a high mortality have been observed in non-irradiated F_1 hybrids after parabiotic junction to a parent strain mouse. This technique allows the rather slow development of a one-directional immunological reaction between non-irradiated animals.

Information obtained from pathological and hematological studies

The results of this part of our investigation will be published in extenso elsewhere but the main findings could not be eliminated in the present discussion.

In mice dying before the 30th day after foreign bone marrow transplantation the bone marrow was the principal site of pathological changes which is in agreement with the concept of a delayed rejection of the bone marrow graft—i.e a host anti-graft reaction—occurring during that period.

After the 4th week pathological changes were found predominantly in tissues other than the bone marrow which excludes bone marrow

failure by a host anti-graft reaction and which is compatible with the assumption of a graft anti host reaction being involved

INTERPRETATION OF THE AVAILABLE INFORMATION

It seems now possible to present an analysis of the various pathological phenomena that follow the transplantation of foreign bone marrow in irradiated mice (text figure 1)

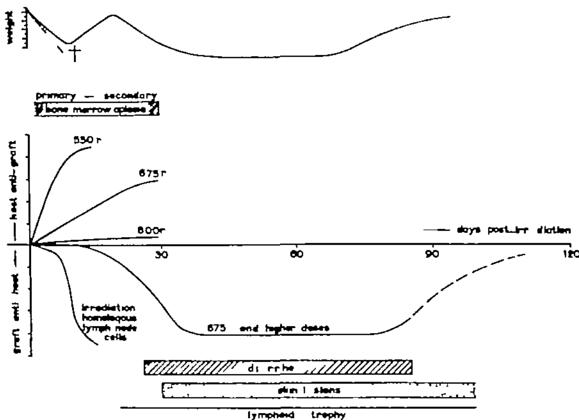


FIG. 1. — *Schematic representation of the relative importance of host anti-graft and graft anti-host reactions at various intervals following foreign bone marrow transplantation in irradiated mice*

The hypothetical magnitude of the host anti-graft reaction and the graft anti-host reaction are shown along the vertical axis in arbitrary units. The main pathological changes are put inside the horizontal bars on the corresponding side of the horizontal axis.

The initial recovery from the radiation induced bone marrow aplasia is dependent on the degree of proliferation of the donor cells. The latter is in turn determined not only by the size of the transplant (number of cells) but also by the residual immunological reactivity of the host. After sublethal irradiaton a proliferation of the grafted cells seems doubtful and early rejection is certain. After lethal doses an initial proliferation of the donor cells may be followed by a secondary rejection and even after a LD_{100} or more some host animals regain the

power to reject a fully established foreign bone marrow (total reversion). When the hosts own hematopoietic tissues recover in time to take over these reversals may survive. In the alternative case death ensues and part of the mortality in the 3rd and 4th week following X ray doses below the LD_{100} is due to this cause. After 800 r very few reversion reactions were observed and none was found to proceed to completion The above description applies to rat bone marrow treated mice

Recently we succeeded in identifying donor and host erythrocytes in the homologous combination and we have not found one single case of total reversal after 675 r[18] This indicates that the delayed host anti-graft reaction is also influenced by the degree of antigenic difference between the host and the donor

The results of Odell and Caldwell[19] are in excellent agreement with the above point of view since long lasting bone marrow grafts were obtained between their closely related C and D rat strains after irradiation with a LD_{50}

The characteristic sequelae of a graft rejection reaction can be summarized as mortality with the highest rate before the 30th day the absence of weight recovery bone marrow aplasia and in the milder forms a high proportion of « total reversals »

Under conditions which favour the proliferation of the foreign cells *(and maximally suppress host reactivity) the animals recover from* the initial bone marrow damage and regain weight rapidly after the 10th day However about the 24th day a secondary weight loss occurs, accompanied by the first signs of diarrhea These symptoms become rapidly more severe and at the end of the first month there is usually *excessive diarrhea and emaciation* At this time skin lesions begin to appear The majority of the animals die with a peak mortality between the 30th and the 70th day The prevalent pathologic finding in this period is colitis, many animals suffer from infections e.g pneumonia and a few cases of focal degeneration of the liver were observed The lymphatic tissues are usually atrophic.

Evidence that the primary cause of the secondary disease is an immunological reaction of the graft against the host has been derived from experiments with F_1-parent *strain combinations by Uphoff Trentin* and by the present authors. The injection of homologous and heterologous lymph node cells is lethal to irradiated mice under the conditions described elsewhere[1] The results obtained with F_1 parent strain combinations suggest that the latter mortality is caused by an *immunological reaction of the grafted* lymph node cells against the host In the animals that died between the 10th and the 30th day after relatively low doses of lymphoid cells clinical symptoms developed which resembled those of the secondary disease *following foreign*

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the work of Feldman and Yaffe[8] These authors results suggested
the production of anti host agglutinins by lymphoid tissue derived from
homologous radiation chimeras. In addition they presented evidence
in support of a cytotoxic activity of the graft directed against the host.
However radiation damage is also an important factor as has been
shown by the results obtained in heavily irradiated CBA mice which
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It should be pointed out that some host anti-graft activity may per
sist after the first month as manifested by delayed graft rejections this
may add to the complexity of symptoms especially during the early
period of secondary disease. Under experimental conditions which do
not completely suppress the early host anti-graft reaction the patho-
logical changes observed during the 4th 6th week may be confusing
since lesions characteristic of both the host anti-graft and the graft
anti-host reaction may be encountered (Fig 1)

Finally there is evidence that a deficient immunological defence
is of considerable significance in secondary disease. The results of
skin transplantations[8 11 12] have shown that the nature of the trans-
plantation immunity in radiation chimeras is determined by the anti
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bone marrow transplantation. Skin lesions and diarrhea were observed in some of these animals. Apparently the transplantation of homologous lymph node cells induces an accelerated development of secondary disease symptoms. Furthermore every one of the characteristic symptoms of the secondary disease has been induced by the provocation of immune reactions directed against non-irradiated mice either by lymphoid cell injections or by parabiosis. Thus accumulated indirect evidence in favour of a graft anti-host mechanism being involved in the secondary disease has recently received additional support from the work of Feldman and Yaffe[8]. These authors results suggested the production of anti-host agglutinins by lymphoid tissue derived from homologous radiation chimeras. In addition they presented evidence in support of a cytotoxic activity of the graft directed against the host.

However radiation damage is also an important factor as has been shown by the results obtained in heavily irradiated CBA mice which were treated with isologous bone marrow as well as in irradiated F_1 mice (after treatment with CBA bone marrow). These findings confirm suggestions made previously by Barnes and Loutit[1] and by ourselves[8]. The early data of both Uphoff and Trentin show a variable incidence of secondary mortality in isologous bone marrow treated mice which seems to be best explained as being due to late radiation damage.

The relative importance of the irradiation and the graft anti-host reaction in the development of the secondary disease symptoms is dependent on the experimental conditions (Table II). Mortality can be caused by each of the two factors, skin lesions are mainly if not exclusively provoked by the immunological reaction and the diarrhea is apparently the result of the combination of the immune reaction and the irradiation.

It should be pointed out that some host anti-graft activity may persist after the first month as manifested by delayed graft rejections; this may add to the complexity of symptoms especially during the early period of secondary disease. Under experimental conditions which do not completely suppress the early host anti-graft reaction, the pathological changes observed during the 4th–6th week may be confusing since lesions characteristic of both the host anti-graft and the graft anti-host reaction may be encountered (Fig. 1).

Finally, there is evidence that a deficient immunological defence is of considerable significance in secondary disease. The results of skin transplantations[4, 12, 13] have shown that the nature of the transplantation immunity in radiation chimeras is determined by the antigenic composition of the foreign bone marrow graft. Taking into account all the other available evidence, the best explanation seems to be offered by the postulate that the immunological system in these

radiation chimeras is of the donor type. However several investigators have also observed that the transplantation reaction against skin that is homologous with regard to the bone marrow donor as well as to the host is usually abnormally slow A severe atrophy of the lymphatic tissue is a characteristic finding in animals suffering from the secondary disease.

TABLE II — *Summary of experimental findings factors involved in production of secondary disease symptoms*

Treatment	Mortality (secondary)	Skin lesions	Diarrhea
Irradiation.	+ only after 950 r in CBA (isologous bone marrow)	—	+ slight after 950 r in CBA (isologous bone marrow)
Foreign graft no irradiation	+ lymph node cells (sensitized donors) parabiosis	+ lymph node cells (sensitized donors) parabiosis	+ lymph node cells (sensitized donors)
Irradiation and foreign bone marrow	+ + heterologous / homologous donors CBA into F (950 r)	+ heterologous / homologous donors	+ + heterologous / homologous donors CBA into F (800 and 900 r)
Irradiation foreign bone marrow and lymph node cells.	+ + + heterologous / homologous donors CBA into F	+ heterologous / homologous donors CBA into F	+ + heterologous / homologous donors CBA into F_1

It seems of interest in this respect that a high incidence of localized infections has been noted in animals suffering from secondary disease in this as well as in other laboratories Furthermore we have recently found that continuous treatment with an antibiotic (aureomycin) significantly inhibits the diarrhea and decreases the secondary mortality [17]

At present it is not known why the diarrhea and the mortality gradually diminish after the 70th day In the rat bone marrow treated groups this may partly reflect the relative increase of the number of total reversals, which no longer suffer from secondary disease However this is certainly not the case in our groups treated with homologous bone marrow in which total reversals did not occur It should be recalled that the skin lesions usually do *not* subside after the 100th day except in the total reversals. In our experience most of the 100 day survivors that have retained the foreign bone marrow graft die within the first year after the irradiation

It is possible that the chimeras which show a recovery from the secondary disease are the resistant individuals the mortality sequence thus reflecting a selection process.

Another possibility seems that a graft anti host adaptation gradually develops after the 70th day The small percentage of survivors after this period constitutes a serious technical difficulty in elucidating this problem.

SUMMARY

The available information concerning the pathogenesis of the secondary disease following foreign bone marrow transplantation in irradiated mice has been reviewed.

Evidence has been provided in support of the hypothesis that the secondary disease in foreign bone marrow treated mice is due to the combined effects of late radiation damage, an immunological reaction of the graft against the host and an impaired immunological defence against micro-organisms.

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Bekkum's data it does not occur except at the highest dose levels against which mice can be protected with bone marrow That such an effect is not essential to the occurrence of homologous disease is indicated by the fact that homologous disease can be reproduced by transplantation of parental lymphoid tissue into completely unirradiated but «genetically tolerant» F_1 hybrid mice.

One possible alternative explanation for the late mortality with isologous marrow at the highest irradiation dose level should also be excluded. It is my understanding that this was obtained with marrow from female mice used to protect male mice was it not? Lengerová and Zeleny in Hašek's laboratory (see Hašek's contribution to the Colloquium) have obtained delayed mortality after isologous marrow protection in the C_{57} strain as a result of a graft versus-host reaction based on the sex linked antigen difference. Delayed mortality was obtained with female-marrow into the male, but not with male marrow into female, or male into male. It is therefore possible that the sex-linked antigen difference of the CBA strain may have become manifest as a graft versus-host reaction at the highest irradiation dose level. This possibility can be excluded by the use of marrow from the same sex

VAN BEKKUM In answer to Dr Trentin's questions, nephrosclerosis does not occur during the period of secondary death as defined in my paper It is a much later manifestation of radiation damage in our mice.

Secondly it has never been my intention to suggest that radiation was a factor in the development of *homologous disease* I have put before you evidence supporting the idea that delayed radiation damage is an additional factor determining the occurrence of *secondary mortality* following foreign bone marrow transplantation Our data also indicate that radiation contributes to the development of *one* of the symptoms of the secondary *disease* namely of the diarrhea

I agree entirely with Dr Trentin that the possibility of a female graft versus-male host reaction contributing to the mortality in our isologous CBA bone marrow transplantation experiments has not been excluded However we do not consider this at all a likely explanation in view of Dr Zaalberg's results with skin transplants, which showed the complete absence of a sex effect in our CBA strains. Furthermore in contrast to Lengerová *et al* we have never seen secondary mortality in irradiated C_{57}Bl males (700 r = LD $_{·}$) protected with C_{57}Bl female bone marrow although the sex effect in this strain is very strong indeed (*vide* Zaalberg)

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DISCUSSION

MATHÉ I should like to cite some preliminary results of two experiments which illustrate the complexity of the relative influence of marrow and radiation in secondary disease

In the first one (done with Dr Amiel and M Iran Ba Loc) we injected 10^3 bone marrow cells from adult DBA_2 mice into 40 newborn $C_{57}Bl_6$ mice intravenously after 3 months we found a DBA_2 hemoglobin type (characterised by starch gel electrophoresis) in five of them 3 out of the 40 had a runt disease.

In the second experiment we injected some adult F_1 ($C_{57}Bl \times DBA_2$) with bone marrow and lymphoid cells from $C_{57}Bl$

Some recipients were unirradiated the remainder had been irradiated with a series of doses up to 850 r There seems to be an inverse correlation between irradiation dose and the time of death but only a few of the unirradiated mice have died and these were the ones that received the large number of lymphoid cells.

TRENTIN I would like to congratulate Dr van Bekkum on his excellent presentation Our data are certainly in agreement regarding the great importance of irradiation dose in determining the prevalence of host versus-graft or of graft versus-host reactions.

With regard to the role of late irradiation effect *per se* apart from immunological effects certainly such delayed effects as intestinal fibrosis and nephrosclerosis are well known Delayed mortality in mice protected against very high doses of irradiation by completely isologous marrow would indicate some such effect which must be separately distinguished from homologous disease of a graft versus host nature Such an effect might increase mortality in mice suffering from homologous disease However at the irradiation dose levels at which I and most other investigators have studied the « late disease such mortality does not occur in mice protected with isologous marrow Even in Dr van

Bekkum's data it does not occur except at the highest dose levels against which mice can be protected with bone marrow. That such an effect is not essential to the occurrence of homologous disease is indicated by the fact that homologous disease can be reproduced by transplantation of parental lymphoid tissue into completely unirradiated but «genetically tolerant» F_1 hybrid mice.

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An analysis of the Eichwald Silmser effect

O Brocades Zaalberg.

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In 1955 Eichwald and Silmser[2] observed that skin grafts from male donors to female recipients were frequently rejected in the A/Jax inbred strain of mice and completely unsuccessful in the C57BL strain In all other sex combinations of donor and recipient the skin grafts were uniformly accepted in both strains.

Other investigators found the same phenomenon to occur in several other strains of mice for instance the A/Fa the C57BL/Fa and the CBA/Fa albeit in the latter strain with a lower incidence.

Various postulations have been presented to explain this effect (Hauschka[4] Snell[11] Eichwald et al [3]).

1. Male skin requires androgene hormones for indefinite maintainance.
2. A linkage of an antigen carried on the non pairing segment of the Y-chromosome.

These hypothesis however did not include an explanation for the strain difference in the occurrence of this phenomenon. To explain this strain difference three possibilities were proposed by Michie and McLaren[8]

1. Strain differences in an Y linked histocompatibility gene or genes and hence in Y controlled antigens.
2. Strain differences in respect of the donors capacity to give effect to the male specific antigen or of his skin s capacity to survive the immune response of the recipient
3. Strain differences in respect of the female recipient s capacity to respond to the male-specific antigen.

We are now able to rule out some of these possibilities The two strains maintained at our laboratory a C57BL and a CBA strain are

particularly suited for this kind of investigation because our C57BL mice show the sex effect to a high degree, whereas in the CBA animals it is completely absent. The data of Table I show that the sex of the donor has no effect on isologous grafts in the CBA mice and a marked effect in the C57BL and the F_1 (CBA ♂ × C57BL ♀) and $F_1{}^1$ (C57BL ♂ × CBA ♀) hybrids.

In the survival times of the individual male C57BL skin grafts on the female hosts there was however a large variation. Both acute and long drawn out reactions up to 90 days were observed. In most cases there was no visible hairgrowth while the graft shriveled up without any signs of inflammation. Microscopic observation *in situ* of some of these grafts however showed that during this period a transient vascular dilatation occurred several times. In the cases of F_1 (CBA ♂ × C57BL ♀) hybrid females grafted with male parent skin and skin from hybrid males there was never any evidence of an acute rejection of the kind that was sometimes observed with the C57BL females.

The intolerance to male skin seems to be inherited by the F_1 and $F_1{}^1$ females although its expression is somewhat weaker in these animals than in the C57BL parent. To examine whether an accelerated break down reaction could be elicited in these hybrids, thirteen of the animals which had sloughed both the CBA and C57BL skin graft (Table I exp nr 3) received a piece of CBA and C57BL male skin for the second time (Table II exp 2 and 2 A). Thereafter having rejected their second pair of grafts these animals received skin from F_1 hybrid males (Table I exp nr 4 Table II exp 2 B) which grafts were also rejected. Two female hybrids (exp nr 3 Table I) retaining both the CBA and C57BL male skin grafts for two months received an additional graft from an F_1 male mouse. A third F_1 female hybrid having only rejected the CBA skin also received an F_1 male skin. All these F_1 male skin grafts were rejected in about 50 days. The CBA and C57BL grafts on these animals from that time on lost their hair and were vanished 295 days after transplantation.

In Table II the mean survival times of the consecutive grafts are presented. It should be mentioned that the transformations leading to an atrophy of the graft were in most cases so gradual that it was difficult to arrive at an exact evaluation of the survival time. The rejection of the graft was reckoned to be complete when only scar tissue was left.

The shortening of the survival time of the consecutive grafts is suggestive for an immunity reaction.

The rejection of the CBA and the F_1 male skin by the F_1 female indicates that these tissues give rise to an immunity reaction and therefore both possess sex linked antigens (Fig 1 and 2). The question rises whether these antigens are identical in both the C57BL and CBA

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CBA

C57BL

♀ ♂
xx xy

♀ ♂
XX XY

F_1

F_1

♂ ♀
Xy Xx

♂ ♀
xY Xx

FIG. 1 — The combinations of sex chromosomes in the F and F hybrids

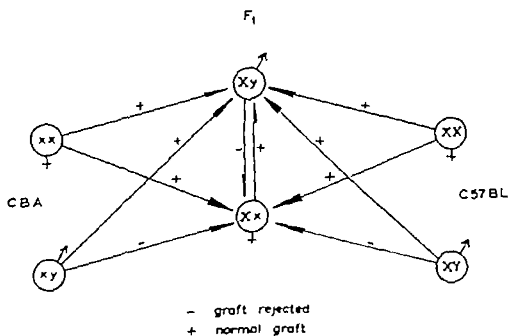


FIG. 2 — Scheme of the host-donor combinations investigated

male. By the following experiments these sex linked antigens were indeed shown to be identical

TABLE I — *The results of skin transplantation in several host-donor combinations*

Exp nr	Donor	Host	Number of takes/Number of experiments			
			♂ to ♂	♀ to ♀	♀ to ♂	♂ to ♀
1	C57BL	C57BL	5/5	6/6	11/11	0/29
2	CBA	CBA	4/4	2/2	26/26	27/27
3	CBA and C57BL	P	40/40	13/13	14/14	2/21
4	F_1	P	40/40	13/13	7/7	3/23
5	F_1^1	P	—	19/19	—	2/26
6	P	F_1^1	—	—	—	8/9
			—	—	—	8/9

) Both CBA and C57BL skin were grafted on the same animal
) Thirteen of the 26 F females grafted with male skin are from exp nr 3
) Due to a technical failure it was impossible to evaluate all the C57BL skin grafts.

The F_1 and F_1^1 (CBA ♀ × C57BL ♂) males genetically differ both in the X and Y chromosome. The absence of an immunity reaction 126 days after cross-grafting on twelve animals is highly suggestive not only of the previously postulated identity of the male antigen in these two strains but also of our hypothesis that these two strains do not differ in respect of histocompatibility loci carried on the non pairing segment of the X chromosome Moreover we were able to immunize C57BL females with CBA male spleen cells which resulted in a shortening of the survival time of the C57BL male skin grafts

In this experiment C57BL females received CBA male spleen cells (10^7/mouse) intraperitoneally 31 28 22 18 14 and 8 days before grafting of C57BL male skin. As controls 9 animals received CBA female spleen cells. The C57BL females that received CBA male spleen cells all sloughed the C57BL male skin 21 44 days after grafting The control group sloughed the skin 50-86 days after grafting This is highly suggestive of an active immunity state in the first group and an additional argument for the hypothesis that the sex linked antigens are not

CBA

C57BL

♀ ♂
xx xy

♀ ♂
XX XY

F_1

F_1'

♂ ♀
Xy Xx

♂ ♀
xY Xx

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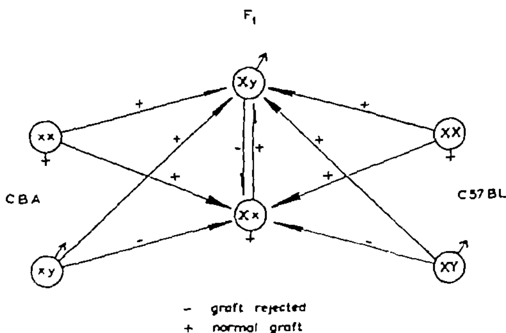


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			40/40	13/13	7/7	3/23
4	F	F	—	19/19	—	2/26
5	F_1	F^1	—	—	—	3/9
6	P	F	—	—	—	3/9

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DISCUSSION

KLEIN I wonder whether the Y antigen may not be something different in principle from the «ordinary» histocompatibility factors, since it seems to be identical in different strains. Could it be an obligatory as opposed to variable, character related for example, to the differentiation of maleness ? Do I understand correctly that the Y chromosome seems to be essential for the development of a male in the mouse as opposed to *Drosophila* ? It should also like to ask whether the rat contains a Y antigen and if so whether this is identical with the Y factor in the mouse ?

OWEN Yes, I believe that W Welshons and L. Russell of Oak Ridge have in press a paper indicating that XO in the mouse is a fertile female that is it seems that the Y chromosome here is a positive male-determining element and that sex determination in mice unlike *Drosophila* is probably not a simple matter of X chromosome-autosome balance I agree with Dr Klein that «male antigenicity» may well be less directly derived from any particular chromosome or locus than we may assume for such an antigen as H-2 for example a material unique to a male may relate to a complex developmental pathway in which the Y chromosome affects an early divergence but the Y need not control directly the specificity of all the ultimate consequences of this divergence Different autosomal genes may for example, be activated over these divergent pathways. I do not know anything about sex linked antigens in rats.

ZAALBERG We could never demonstrate a sex effect in our inbred Wistar-Glaxo rat strain This does not prove that there is no sex linked

antigen in our rats because our negative finding could be due to the fact that our female rats do not react against the male-specific antigens—as is the case in our CBA strain

BRENT I believe that Billingham and his colleagues have recently shown that tolerance induced on behalf of the sex linked antigens of one strain will also extend to the sex linked antigens of another strain These results have not yet been published but they support very strongly Dr Zaalberg's conclusion that the tissue antigens on the Y-chromosome are not strain-specific.

ZAALBERG I am interested to know of Billingham's results because together with ours, they suggest that the lack of strain specificity of sex linked tissue antigens may be a general phenomenon

SIMONSEN Billingham and Silvers have recently drawn attention to the fact that an inbred strain which displays the immunological Y-chromosome effect is comparable to a pair of co-isogenic strains In fact, it is probably more strictly co-isogenic than the artificially produced IR lines of Dr Snell. It seems to me therefore, that a cell free antigen preparation made from male tissue of a strain with marked Y-chromosome effect would be the ideal preparation to use for the investigators who are trying to determine whether or not immunological tolerance can be achieved with a soluble antigen If such a preparation can initiate immunity but not induce tolerance, the negative result is really significant.

ZAALBERG I think that your suggestion is a very good one, and it is to be hoped that an experiment on these lines will be carried out

Transfusion (*) et greffe (*) de cellules myéloïdes chez l'homme

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Les recherches expérimentales sur la souris et d'autres animaux de laboratoire ont montré qu'il était possible de greffer la moelle osseuse[6, 10, 13, 11, 23, 12, 11, 11, 35] et ont permis l'étude de quelques applications thérapeutiques particulièrement aux leucémies[7, 11, 34, 35, 48].

Ces recherches ont établi certaines conditions de la greffe : *a*) le receveur doit être isologue par rapport au donneur ou s'il est homologue conditionné par une irradiation transcutanée totale (rayons X ou γ ou éventuellement, neutrons[45] à dose très élevée : classique dose 100 % létale) ; les essais de conditionnement par les hormones cortico-surrénales, les radiomimétiques chimiques semblent inefficaces[46, 29] ; *b*) un nombre minimum de cellules myéloïdes vivantes doit être administré (10^6 à 10^6 pour une souris de 15 g si la greffe est isologue ; 10^7 à 10^7 si la greffe est homologue) ; *c*) l'injection doit être veineuse.

On dispose, pour démontrer la prise d'une greffe de moelle de plusieurs types de méthodes (chimiques, sérologiques, etc.). La greffe peut être définitive ou transitoire.

Les animaux irradiés et restaurés par une greffe de moelle homologue sont fréquemment atteints d'une « maladie secondaire » dont le mécanisme encore obscur est peut-être variable d'un cas à l'autre mais semble pouvoir être lié à une immunisation du greffon contre l'hôte (maladie homologue[9, 21, 3, 31, 33, 41, 44]).

(*) Nous respecterons la terminologie suivante : on utilisera le terme de transfusion de moelle pour désigner l'injection veineuse de celle-ci sans préjuger de son résultat. On dira que la greffe de cette moelle (tissu dont la fonction essentielle réside dans la production de cellules) « a pris » lorsque l'on pourra démontrer la survie, la maturation et la division des cellules administrées. Rappelons enfin que le terme de transplantation est réservé aux greffes d'organes avec établissement de connections vasculaires[6].

Malgré de nombreuses inconnues, il n est pas déraisonnable que des équipes spécialisées commencent à tenter d appliquer prudemment cette méthode chez des humains atteints d affections rapidement mor telles [1, 2, 14, 17, 21, 24, 27, 39, 41]

Nous avons fait des transfusions de moelle chez 22 humains.

I. — Technique [37]

Préparation du receveur En cas de greffe isologue, aucune prépa ration n est nécessaire [34]

En cas de greffe homologue, le seul procédé de conditionnement actuellement connu comme efficace est l irradiation transcutanée totale du corps aux doses susceptibles d'inhiber les défenses immunitaires. Les rayons X émis sous 200 Kv ne semblent pas convenir chez l'homme à cause de leur insuffisante pénétration Les rayons X émis sous haut voltage (*) les rayons γ (*) les neutrons (*) sont plus satisfaisants puisque de pénétration plus forte On ne doit pas seulement tenir compte de la dose reçue à la peau il convient de calculer la dose en rad (**) ou en rem (**) et sa répartition

Choix du donneur En cas de greffe isologue le donneur est le jumeau univitellin du receveur

En cas de greffe homologue, on choisit un donneur de même phénotype de groupe sanguin ou de phénotype voisin ne différant que par un caractère très peu antigénique (***)

Le prélèvement de la moelle est d autant plus facile que le donneur est plus maigre et moins musclé On s assure qu il n est porteur d au

( ) Nous remercions MM. Jacques Bernard, B Pierquin, Cl. Lalanne et Mme Dutreix pour la contribution qu'ils ont apportée à ce travail.

( ) Rappelons que 1 *roentgen* est la quantité de rayons X ou γ telle que l'émission d électrons provoquée par elle dans 1 cm d'air sec à 0°C et 760 mm. de Hg (0 001293 g) forme des ions des deux signes transportant une quantité d'électricité égale à une unité électrostatique. Un r correspond à une absorption d'énergie de 83 7 ergs dans 1 g d air c'est-à-dire à 1,61 × 10^{12} paires d'ions. Cette unité roentgen vaut seulement dans l'air dans les milieux différents de l'air la quantité d'énergie absorbée par unité de volume pour la même quantité de rayonnement est différente (dans les tissus mous l'énergie absorbée par g de tissu et par roentgen est d'environ 93 ergs, dans les os elle peut être supérieure) Cette unité ne s'applique enfin qu'aux rayons X et aux rayons γ

Il faut donc utiliser une unité de dose qui puisse s'appliquer au rayonnement corpusculaire (et non seulement aux rayons X ou γ) qui ne mesure pas seulement une dose d exposition mais qui exprime l'énergie absorbée

Un rad (« radiation-absorbed-dose ») est la quantité de rayonnement qui correspond à une énergie absorbée de 100 ergs/g

Un rem (« roentgen-equivalent-man ») est la dose d'un rayonnement ionisant quelconque qui, administré à un homme est biologiquement équivalente à la dose de 1 roentgen de rayons X ou de rayons gamma.

( ) Une faible différence dans le phénotype de groupe sanguin permettra d'obtenir une preuve directe de l'éventuelle prise de la greffe

cune maladie chronique ou aiguë transmissible (syphilis, hépatite, autres infections, parasitoses sanguines, affection érythrocytaire congénitale).

Prélèvement de la moelle. On ponctionne, sous anesthésie générale avec des trocarts à « ponction osseuse » le manubrium et tous les segments du corps du sternum, une vingtaine de secteurs de l'os iliaque, l'acromion, les côtes, les apophyses épineuses des vertèbres. On aspire de chaque secteur deux fois 5 cm^3 (ou plus) de sang mêlé de moelle [les seringues sont « rincées » avec une solution très diluée d'héparine (*)]. L'aspiration doit être énergique. Une numération des cellules nucléées est faite dans chaque échantillon prélevé; il est utile de vérifier le pourcentage des cellules vivantes par le test à l'éosine[24]; leur taux est habituellement de l'ordre de 80 %.

Le nombre de cellules généralement retiré des divers secteurs est donné sur le tableau I.

TABLEAU I

Os et secteurs	*Nombre de cellules nucléées au mm³*
Sternum	
Manubrium	15 000 à 50 000
Divers segments du corps	15 000 à 50 000
Os iliaques	
Épines iliaques ant. sup.	10 000 à 45 000
Pourtour	10 000 à 40 000
Faces post. et massifs iliaques post.	20 000 à 80 000
Côtes	20 000 à 80 000
Acromions	15 000 à 40 000
Vertèbres	
Apophyses épineuses	10 000 à 80 000

Injection au receveur. Le mélange de sang et de cellules médullaires obtenu est injecté immédiatement au receveur par voie veineuse directement à la seringue et aussi lentement que possible.

Aucun accident n'est survenu jusqu'à présent au moment de l'injection. Des accès de toux peuvent être observés après l'injection d'une certaine quantité de moelle. Un clocher fébrile peut être noté dans les quarante-huit heures qui suivent.

Le nombre minimum de cellules à injecter semble être de 10 à 15 milliards pour un adulte.

(*) L'hyperhéparinémie induite chez le receveur est aisément corrigée par l'administration de sulfate de protamine.

Surveillance du receveur. Des contrôles quotidiens de l'état hématologique de l'hémostase et du fonctionnement des différents viscères et systèmes biologiques de l'organisme sont nécessaires.

Pendant la période d'aplasie médullaire et lymphoïde qui se manifeste en cas de conditionnement par irradiation, un traitement symptomatique doit être appliqué : transfusion de sang pour corriger l'anémie ... les désordres de l'hémostase contre lesquels on peut encore lutter par ... plasma, du fibrinogène, des plaquettes ; exsanguino-transfusion en cas de syndrome hémorragique ; antibiotiques (sans excès cependant) et gamma-globulines pour corriger l'inhibition des défenses de l'organisme contre l'infection.

Si la greffe prend, la surveillance doit être poursuivie pendant très longtemps (plusieurs mois) pour déceler un éventuel syndrome secondaire.

Si la greffe ne prend pas, l'essai peut être théoriquement répété sans nouvelle irradiation (si la dose de celle-ci a été suffisante).

Preuves de la prise de la greffe. Une différence même légère, dans les phénotypes peut fournir une preuve directe de la prise et de la survie de la greffe [29, 47, 66]. Le marquage des érythroblastes du donneur donne une preuve de la tolérance et de la prise mais non de la survie [24]. Si donneur et receveur sont de sexes différents, les caractères morphologiques des leucocytes liés au sexe peuvent être utilisés [18]. Les examens hématologiques fournissent enfin des arguments indirects (qui seront analysés plus loin).

Essais de greffe de cellules embryonnaires. Chez l'animal des restaurations d'aplasie par les rayons peuvent être obtenues par la greffe de cellules myéloïdes embryonnaires [29]. Des études sont en cours dans plusieurs centres pour tenter d'appliquer cette technique à l'homme.

« *Banque* » *de cellules myéloïdes.* Ces cellules embryonnaires sont conservées à —70° selon des conditions rigoureuses [8, 21, 22, 43]. On peut également conserver ainsi des cellules médullaires adultes. Une vérification de la vie de ces cellules après réchauffement [26] est évidemment nécessaire avant de les utiliser pour restaurer un sujet irradié.

II. — OBSERVATIONS

1. *Transfusion de moelle d'un jumeau à un autre. Preuve de sa tolérance* [24].

Nous avons, chez un sujet atteint de leucémie aiguë en ré... complète, fait sans irradiation ni autre essai de conditionneme...

transfusion de moelle osseuse de son jumeau (*) afin *a*) de voir si les cellules administrées seraient tolérées comme dans les conditions de la greffe isologue chez l'animal *b*) de tenter de retarder la rechute.

L'enfant receveur atteint d'ichtyose congénitale, est du phénotype de groupe sanguin suivant O M N (**) S S P+ cde cde kk Fy(a) J k(a) Le (a^-)(b) Son frère jumeau dont la ressemblance semble totale et entraîne la confusion, atteint également d'ichtyose congénitale a le phénotype suivant O M (**) S S P + cde cde kk Fy(a) J k(a) Le (a^-)(b).

On a injecté au leucémique en rémission 212 cm^3 de liquide médullaire de son frère, soit 8 milliards 200 millions de cellules nucléées vivantes.

Dix microcuries de Fe^{59} avaient été injectées au donneur 18 heures avant le prélèvement (pour marquer les érythroblastes). On a mesuré sur plusieurs échantillons la radio-activité de la moelle injectée (4 % environ de la radio-activité totale injectée au donneur) et l'on a étudié pendant plusieurs jours la radio-activite apparaissant dans le sang circulant du receveur et dans celui du donneur

La fraction de la radio-activité injectée présente dans le sang du receveur s'est élevée progressivement de 35 % au lendemain de l'injection (c'est-à-dire au second jour après l'injection de fer au donneur) à 80 % aux 8e et 10e jours suivant l'injection des cellules myéloides

Cette methode nous montre donc que la maturation des érythroblastes injectés et la sortie dans la circulation d'hématies marquées se font normalement (***). Il n'a pas ete observé de chute importante de la radio-activité, suivie de réutilisation retardée comme cela eut été noté en cas de destruction des erythroblastes injectés et réutilisation du fer par la propre moelle du receveur

Il n'est cependant pas possible d'affirmer la multiplication à plus long terme de la greffe seule la maturation erythroblastique des premiers jours pouvant etre étudiée par cette technique

On a observe par ailleurs une discrète élevation des reticulocytes (dont le taux par mm^3 passe de 480 avant l'injection de la moelle à 2 700 le lendemain et 4.000 au 4 jour ce qui correspond à la quantite que l'on aurait pu prevoir à partir du nombre d'erythroblastes injectés)

(*) Ce travail a été fait en collaboration avec Mme Larrieu et MM. Jean Bernard Najean et Schwarzenberg.

(**) Nous dicuterons ailleurs l'absence de l'antigène N chez le second

(***) Nous avons comparé, avec R. Najean, les résultats de ce test chez des souris recevant, sans conditionnement, une transfusion de moelle isologue ou homologue le pourcentage de la radio-activité présente dans le sang aux 3me et 6me jours est nettement supérieur lorsque la moelle est compatible que lorsqu'elle est incompatible (respectivement 24 et 37 4 p 100 en cas de greffe isologue et 18 et 21 p 100 en cas de greffe homologue)

et des polynucléaires dont le pourcentage passe de 54 avant l'injection à 71 les jours suivants, pour se maintenir à 60 pendant une douzaine de jours.

Aucun effet n'a été observé sur la leucémie qui a présenté une rechute mortelle un mois après.

2 *Transfusions de moelle homologue*

A) *Echecs chez les receveurs non conditionnés par une irradiation totale à dose élevée*

Nous avons fait des essais chez des sujets aplasiques et des patients leucémiques en pleine phase évolutive dans les conditions suivantes transfusion de la moelle de la mère à l'enfant sans conditionnement transfusion de la moelle d'un donneur de phénotype aussi voisin que possible à des enfants non conditionnés ou traités par les hormones cortico-surrénales à doses moyennes ou fortes (500 mg de cortisone par jour) ces transfusions de moelle n'ont été suivies d'aucun effet (six patients)

Nous avons ensuite soumis d'autres enfants leucémiques (6 en tout) à une irradiation X totale sous 200 kv il s'agissait de nourrissons les conditions de pénétration étaient donc telles que l'irradiation a pu être presque homogène les doses ont varié de 100 à 600 roentgen peau selon les sujets. Deux autres enfants ont été irradiés au bêtatron à des doses respectives de 400 et 500 rad

Ces irradiations ont été fractionnées (en 3 à 4 séances échelonnées sur 2 à 4 jours) La moelle injectée était un mélange de moelle prélevée sur plusieurs donneurs (dont les phénotypes de groupes sanguins étaient aussi voisins que possible de ceux des receveurs)

Aucun de ces essais n'a été suivi de prise de greffe. Trois faits sont à noter *a*) l'apparition dans les jours qui ont suivi l'injection de la moelle d'une polynucléose relative transitoire (durant quelques jours) *b*) l'apparition dans le sang de cellules hyperbasophiles (plasmocytes et proplasmocytes) *c*) la tolérance de l'aplasie médullaire et lymphoïde pendant une dizaine de jours seulement après cette période surviennent chez les leucémiques des complications mortelles Dans le cas de ces patients les parts respectives de l'aplasie et de la leucémie à l'origine de la mort sont impossibles à déterminer (tous ces sujets étant en pleine phase évolutive terminale lorsque l'essai a été entrepris)

A noter que le sujet irradié à la dose de 500 rad au bêtatron présentait à l'examen histologique une aplasie totale de la leucose et des cellules hématopoïétiques myéloïdes et lymphoïdes

B) *Essais chez des humains irradiés à très fortes doses*

1) *Sujets non leucémiques*

Nous avons eu l'occasion de faire des transfusions de cellules myéloïdes homologues chez 5 sujets irradiés accidentellement (*). Les détails concernant l'accident, l'étude quantitative de l'irradiation, l'expression clinique et biochimique du *mal des rayons chez ces patients* seront décrits dans un autre article [21].

L'irradiation accidentelle a eu lieu le 15 octobre 1958 à Belgrade, touchant six physiciens qui réalisaient une expérience à l'aide d'un réacteur à puissance O : celui-ci, dépassant son point critique, a émis des neutrons et des rayons gamma.

Les doses de neutrons ont pu être estimées (**) d'une part par la radio-activité *induite (sodium)*, *d'autre part par l'étude de l'activation* des objets portés par les sujets pendant l'expérience.

On a pu de même estimer les doses de rayons gamma reçues grâce aux données enregistrées par plusieurs dosimètres situés à différentes distances du réacteur. On peut également les prévoir par calcul à partir des doses de neutrons émises (on a choisi pour ce calcul le facteur 3). Les données concernant cette irradiation sont consignées sur le tableau II.

Les *résultats de cette estimation ont été connus vers le 25 octobre.* Les désordres hématologiques observés (***) tendaient à confirmer les notions acquises par les mesures physiques.

Un traitement symptomatique a été institué, comportant des transfusions de sang ou de culots érythrocytaires (destinées à éviter une anémie *trop accentuée), des transfusions de plaquettes isolées (devant la* menace d'un syndrome hémorragique), des antibiotiques et des gamma globulines (pour pallier l'insuffisance leucocytaire). Le maximum d'asepsie a été assuré, une diététique convenable alimentaire et médicamenteuse (vitamines A, B, C, K, PP) instituée [22].

La cytopénie sanguine fut tolérée jusqu'au 25e jour environ après l'irradiation.

Devant l'aggravation assez rapide de l'état de V, *un essai de greffe* de cellules myéloïdes embryonnaires fut décidé. Le patient reçut le 29 octobre 4 milliards 760 millions de cellules libres nucléées extraites de la rate et du foie d'un prématuré mort quelques heures après sa nais-

(*) Ce chapitre est extrait de l'article réf. 20 : G. Mathé, H. Jammet, B. Pendić, L. Schwarzenberg et coll., actuellement sous presse pour paraître dans la *Rev. Fr. Ét. Cliniques et Biologiques* 1959, 4, n° 3. Nous renvoyons à cet article pour les figures résumant les données hématologiques.

(**) Nous remercions particulièrement MM. H. Jammet et L. Jeanmaire pour leur collaboration dans cette étude.

(***) Les hémogrammes ont été établis dans les laboratoires de MM. J. L. Duplan et B. Maupin.

Les myélogrammes du 30 octobre 1958 révèlent une aplasie totale.

TABLEAU II — *Données concernant l'âge le sexe et l'irradiation.*
(G Mathé, H. Jammet, B Pendic, L. Schwarzenberg et coll, Rev Fr Et. Clin. Biol., 1959 4 n° 5)

	Sexe & âge	(1) Na² μ c	(2) Doses de neutrons rem	(3) Doses de rayons gamma estimées à partir de celles des neutrons rem	(4) Doses de rayons gamma estimées par les données enregistrées par les dosimètres rem	(5) Doses totales calculées à partir de (2) et (3) rem	(6) Doses totales estimées à partir des conditions de l'irradiation et des données cliniques et hématologiques rem
V	m 25 ans	82	210	630	450 1000	840	1000 1200
M	m 27 »	73	214	642	450 1000	856	700 1000
G	m 25 »	76	230	690	450 1000	920	700 1000
D	f 24 »	63	256	768	450 1000	1024	700 1000
H	m 23 »	53	174	522	450 1000	696	600 800
B	m 34 »	45	102	306	250 500	408	300 500

sance, d'âge fœtal de 5 mois, le prélèvement des organes ayant eu lieu quelques minutes après la mort. Ces cellules furent injectées par voie veineuse sans incident. Aucun résultat clinique ni hématologique ne fut observé dans les jours qui suivirent. Le 7 novembre 1958 l'état du patient s'aggrava par l'apparition d'un syndrome intestinal s'exprimant par une douleur spontanée et provoquée dans la fosse iliaque droite ainsi que par une impression de plastron perçue à la palpation. La décision fut alors prise de lui faire une transfusion de cellules médullaires prélevées chez un donneur volontaire adulte (tableaux III et IV). Celle-ci fut suivie, quatre jours après, d'une ascension rapide des cellules sanguines. Durant ce temps, néanmoins, les signes intestinaux s'accentuèrent et le syndrome se compliqua d'anurie avec les signes cliniques et humoraux de l'insuffisance rénale aiguë. A la fin d'une épuration extrarénale par le rein artificiel (Service J. Hamburger, Hôpital Necker) le sujet mourut d'une importante hémorragie du tractus respiratoire.[31]

L'aggravation de l'état des autres sujets irradiés à forte dose (M, G, D et H) a également conduit à la décision d'une transfusion veineuse de moelle osseuse prélevée identiquement chez des donneurs adultes, un donneur pour un receveur et du même sexe (tableau III et IV).

TABLEAU III. — *Transfusion de cellules médullaires de donneurs adultes chez cinq des sujets irradiés*

Date, volume de liquide médullaire, nombre de cellules nucléées injectées

(Extrait de G. Mathé, H. Jammet, B. Pendic, L. Schwarzenberg et coll., *Rev. Fr. Et. Clin. Biol.*, 1959, 4, n° 3.)

Receveur	*Date*	*Volume de liquide médullaire*	*Nombre de cellules nucléées injectées*
V	11 nov. 1958	211 cm	$8{,}5 \times 10^9$
M	11 nov. 1958	123 cm	11×10^9
G	17 nov. 1958	270 cm	12×10^9
D	17 nov. 1958	300 cm	$8{,}5 \times 10^9$
H	20 nov. 1958	300 cm	14×10^9

Les états hématologiques et cliniques des quatre sujets ainsi traités évoluèrent dès lors parallèlement dans un sens favorable. On assista à une ascension assez rapide des taux de réticulocytes, de granulocytes et de plaquettes. Puis la concentration sanguine des hématies s'est élevée. Une remarquable amélioration de l'état clinique fut observée tandis

que remontait le taux des granulocytes chute de la température à la normale, disparition du tuphos qui s'était installé quelques jours auparavant reprise de l'appétit gain de poids ».

TABLEAU IV — *Comparaison des phénotypes de groupes sanguins des quatre couples donneur receveur*

(Extrait de G Mathé H. Jammet, B. Pendic L. Schwarzenberg et coll. *Rev Fr Et. Clin. Biol.*, 1959 4, n° 5.)

1 *Malade* M.	O	MN	CCDee	kk	Fy (a +)	Jk (a +)	Le (a — b —)
Donneur P	O	M	CcDee	kk	Fy (a +)	Jk (a —)	Le (a — b +)
2 *Malade* D (fem.)	O	MN	ccDE	kk	Fy (a +)	Jk (a +)	Le (a — b —)
Donneuse D (fem.)	O	MN	CcDee	kk	Fy (a —)	Jk (a —)	Le (a — b +)
3 *Malade* G	O	MN	CCDEe	kk	Fy (a +)	Jk (a +)	Le (a — b +)
Donneur B.	O	M	CCDee	kk	Fy (a +)	Jk (a +)	Le (a + b —)
4. *Malade* H.	A 2	M	ccDE	kk	Fy (a +)	Jk (a +)	Le (a — b —)
Donneur B.	A 2	M	CcDee	kk	Fy (a +)	Jk (a +)	Le (a + b —)

L'étude des phénotypes de groupes sanguins selon la méthode d'agglutination différentielle type Ashby modifiée selon les techniques de Wurmser (*) a permis d'établir les pourcentages respectifs des hématies appartenant aux phénotypes des donneurs et de celles qui portent les antigènes propres aux receveurs Une cause d'erreur liée *a*) aux transfusions de sang antérieures à l'injection de moelle, *b*) aux hématies du donneur de moelle administrées en même temps que les cellules nucléées doit être prise en considération le phénotype de groupe sanguin des donneurs de sang n'étant pas connu pour chacun d'eux, on a calculé la quantité maxima d'hématies qui aurait été administrée si les donneurs de sang de chaque receveur portaient le (ou les) caractères antigéniques du donneur de moelle qui a (ou ont) servi à l'identification de sa population cellulaire (**) On a supposé que les hématies introduites par ces transfusions avaient une durée de vie de 100 jours

(*) Étude faite sous la direction de M. Ch Salmon, chef de laboratoire au Centre Régional de Transfusion Sanguine Hôpital Saint-Antoine Paris et avec l'aide de M. L. Schwarzenberg, chef de laboratoire à l'Hôpital Saint-Louis, Paris

(**) On notera que pour D les chiffres de la population érythrocytaire caractérisée par le phénotype du donneur de moelle sont inférieurs à ceux calculés comme pouvant être fournis par les transfusions, ce qui montre étant donné l'ascension de la courbe et son parallélisme avec celle des hématies, l'exagération certaine de l'estimation de l'erreur que les transfusions ont pu induire

(ce qui apparaît très exagéré puisque les transfusions n'ont pas été suivies de l'ascension du taux des erythrocytes, que l'on aurait pu attendre si cette dernière condition était réalisée)

On observe *a*) que les courbes du ou des antigènes propres à chaque donneur s'élèvent pour atteindre un maximum puis commencent à descendre un mois environ après la transfusion de moelle *b*) que pendant la période d'ascension, ces courbes sont pour chaque receveur parallèles à celles des concentrations sanguines des hematies la correction de l'anémie semble donc entièrement liée à la production érythrocytaire du greffon.

Les aspects quantitatifs et qualitatifs des myélogrammes établis chaque semaine à partir du 29 novembre sont normaux pour l'un d'entre eux, proches de la normale pour deux qui se caractérisent par une augmentation discrète et transitoire des cellules hyperbasophiles (G et M) accompagnée chez M d'une éosinophilie à 20 %

Dans un second temps, entre le 5 et le 12 décembre pour M qui reçut la moelle le 11 novembre entre le 12 et le 19 pour G D et H qui reçurent la moelle le 17 ou le 20 on assiste à une dissociation des deux courbes a et b celle du taux de la population sanguine des descendants des cellules greffées tend à baisser tandis que celle des hématies totales présente une nouvelle phase ascendante. Il est raisonnable de considérer que reprend à ce moment la propre production érythrocytaire du receveur (*) Les tests de Coombs pratiqués entre le 28 novembre et le 14 février chez chacun des sujets sont négatifs.

Quelques semaines après, l'on peut observer de discrètes oscillations des courbes leucocytaires et thrombocytaires (entre 7.000 et 2.000 pour les premières 200.000 et 120.000 pour les secondes) Bien qu'aucun caractère ne permette de distinguer pour chaque patient les deux populations respectives du donneur et du receveur il n'est pas déraisonnable de se demander si ce léger désordre ne correspond pas au changement des populations cellulaires issues des cellules souches des donneurs par celles propres aux receveurs.

Les états cliniques et hématologiques sont satisfaisants à la date du 15 février 1959. L'état des patients sera contrôlé à partir de cette date à Belgrade.

Le lecteur trouvera dans une autre publication ** les commentaires et discussions concernant les doses reçues les divers symptomes cliniques hematologiques, biochimiques observés les traitements symptomatiques administrés.

La presente discussion est limitée à la greffe de moelle osseuse et à son rôle thérapeutique.

(*) C'est également à cette période qu'a repris la croissance des cheveux

La transfusion de cellules myéloïdes embryonnaires chez le patient le plus irradié mérite deux remarques *a*) l'on ne saurait affirmer que celle-ci n'a pas été et n'aurait pas été suivie de « prise » de greffe, un recul de temps suffisant manquant à cet égard une greffe de cellules myéloïdes peut ne commencer à corriger la cytopénie sanguine qu'après un délai de dix jours, *b*) la quantité de cellules nucléées administrée est de 4 milliards alors qu'elle est de 8 à 14 milliards pour les cellules médullaires de donneurs adultes il est possible que le premier chiffre ait été insuffisant (*) *c*) des observations recueillies dans d'autres essais ont conduit à distinguer dans le résultat d'une greffe de moelle de donneur adulte un effet rapide de maturation des cellules les moins immatures et un effet secondaire, d'expression plus tardive, dépendant de la repopulation du tissu myéloïde par les éléments souches formés et conditionné par la « prise » de greffe de ceux-ci ou d'une certaine proportion d'entre eux, leurs divisions au stade de cellules souches leur engagement dans telle ou telle lignée sanguine, leur différenciation leurs divisions maturatrices. Peut-être l'effet rapide de maturation n'a-t-il pas lieu avec les cellules embryonnaires qui, pour la plupart d'entre elles, sont des cellules souches

La distinction de ces deux effets n'apparaît d'ailleurs pas chez les sujets de la présente étude, aussi clairement que dans d'autres observations. On peut supposer que, la moelle de donneurs adultes ayant été injectée longtemps après l'irradiation, en pleine phase cytopénique, les mécanismes d'hyperplasie compensatrice ont été au maximum

En faveur de la « prise » de greffe de la moelle des donneurs adultes, on peut inscrire des arguments indirects et un argument direct.

Parmi les premiers mentionnons *a*) la soudaineté, après la transfusion de la moelle, de l'ascension des taux sanguins des réticulocytes, polynucléaires et plaquettes ainsi que la rapidité de cette ascension *b*) la dissociation nette entre les courbes des polynucléaires qui montent rapidement et intensément et celles des lymphocytes (**) qui sont peu influencées par la transfusion *c*) l'aspect des courbes des plaquettes qui montent rapidement pour atteindre un « pic » (surtout chez V et M) pour redescendre ensuite à une concentration un peu inférieure et assez stable *d*) l'ascension des taux des cellules sanguines s'est produite, chez tous les sujets quelques jours après la transfusion de moelle

(*) On sait que chez l'animal, un nombre minimum de cellules est indispensable pour obtenir la restauration dans nos essais, ce nombre est plus élevé pour les cellules myéloïdes embryonnaires que pour les cellules médullaires de donneurs adultes.

(**) Il convient de mentionner la difficulté de distinguer les lymphocytes vrais d'autres cellules mononucléées, en particulier les monocytes dont le taux s'élève après la transfusion de moelle il est donc possible que les chiffres des premiers soient légèrement exagérés par une telle confusion.

et non un nombre de jours déterminé et identique pour tous après l'irradiation or si l'irradiation des six patients a été simultanée, les transfusions de moelle ont été faites entre le 11 et 20 novembre *e*) la comparaison des courbes des sujets ayant reçu de la moelle osseuse avec celles du patient non traité par cette méthode ce dernier ne présente pas de « crise » réticulocytaire le taux de ses polynucléaires ne monte pas d'emblée et rapidement mais seulement lentement et n'atteint son taux le plus élevé que 50 jours après le moment de la cytopénie maxima (*) sa concentration sanguine de plaquettes ne dépasse guère 200.000 par mm³ et leur courbe ne présente pas de pic; l'ascension générale des taux des diverses cellules sanguines est beaucoup plus lente que chez les sujets ayant reçu de la moelle et pourtant la cytopénie maxima observée chez ce patient a été très inférieure à celle des malades traités (2.000 leucocytes par mm³) Ajoutons que l'état clinique s'est amélioré soudainement chez ces derniers après l'injection de la moelle, beaucoup plus lentement chez ce patient

Notons enfin que de nombreuses expériences faites sur l'animal [12] ont bien montré que des cytopénies modérées comme celle de ce sujet sont corrigées spontanément et rapidement, tandis que les cytopénies sévères comme celles observées chez G D H et M n'ont pas tendance à se corriger aussi rapidement et conduisent en règle générale à des complications mortelles

Une preuve directe de la « prise » de greffe est enfin apportée par l'identification grâce au phénotype de groupe sanguin des hématies produites par la moelle greffée.

On peut voir *a*) que les concentrations de ces hématies dans le sang s'élèvent pendant le premier mois *b*) qu'elles dépassent les taux que pourraient avoir apporté les transfusions de sang *c*) que le nombre total d'hématies produites par la greffe est considérablement supérieur au nombre d'érythroblastes administrés, ce qui prouve qu'en dehors des phénomènes de survie de maturation a eu lieu un phénomène de division cellulaire et singulièrement de division maturatrice puisque productrice de cellules sanguines

Nous ne discuterons pas ici la validité de la méthode utilisée entre les mains de techniciens entraînés, elle permet d'obtenir des résultats reproductibles l'erreur est estimée à ± 5 % [1 **] L'évolution des taux des cellules produites par la greffe qui présentent une courbe ascendante puis descendante le parallélisme entre les courbes des divers sujets irradiés et traités de façon semblable l'étroite superposition entre les courbes obtenues chez le même patient lorsqu'on a disposé de deux

(*) On notera que chez ce sujet, le taux des lymphocytes par mm³ s'est élevé (au-dessus de 1000) un peu avant le moment de la granulopénie maxima

antigènes (en particulier chez G E et Lewis a ce dernier etant le plus susceptible de cause d erreur) soulignent la validité de cette méthode.

La prise de greffe semble donc prouvée.

Il convient de discuter son rôle thérapeutique On sait que chez l animal une irradiation totale aux doses reçues par G D H et M est mortelle dans 100 % des cas. Il semble certain que la réascension des taux des cellules sanguines n aurait pas eu lieu chez eux au moment ou la greffe a permis de l obtenir au moment ou elle a eu lieu chez le sujet non traité, car celui-ci ne présentait qu une cytopénie modérée n ayant reçu qu une dose d irradiation inférieure (environ la moitié)

Ajoutons que l'identification des deux populations d erythrocytes produits respectivement par la greffe et par le receveur lui même indique que la production cellulaire de celui-ci n a eu lieu qu un mois après l ascension liée à la greffe. Or c est précisément à ce moment que les cheveux ont réapparu, autre indication de l absence de division des cellules des patients entre l'irradiation et le 20 décembre environ.

Ajoutons que l ascension des taux d'hématies après la transfusion de moelle est parallèle, pour chaque patient à celle des taux de globules rouges produits par la greffe autre indication que la seule production cellulaire a eté pendant un mois, due au greffon

On a toutes raisons de penser que les productions leucocytaires et thrombocytaires ont eu la même origine Mais, si les taux de globules rouges avant la transfusion de moelle n étaient pas effondrés car maintenus facilement par des transfusions sanguines à des chiffres satisfaisants, les concentrations granulocytaires et thrombocytaires étaient effondrées il est donc très raisonnable de penser que si l ascension des taux erythrocytaires est entièrement due à la greffe, le rôle thérapeutique de celle-ci est beaucoup plus important pour les granulocytes et les plaquettes, la population entière lui étant due puisque les chiffres au moment de la transfusion de moelle étaient effondrés. Ajoutons que la durée de vie des granulocytes et des plaquettes étant considérablement plus courte que celle des hématies, la quantité de ces cellules produites a dû être très supérieure à celle des érythrocytes.

On remarque encore, en comparant les courbes des quatre patients qu'il semble exister une compétition entre les ascensions respectives des hematies d une part des granulocytes et des plaquettes de l autre chez D qui était notablement anémique (et dont la stimulation des compensations érythrocytaires a dû être importante) le taux d'hematies monte relativement plus vite que ceux des granulocytes et des plaquettes le contraire est observé chez G qui n était pas anémique et chez M qui l était très peu D qui présentait une anemie moderee, reagit de façon intermédiaire.

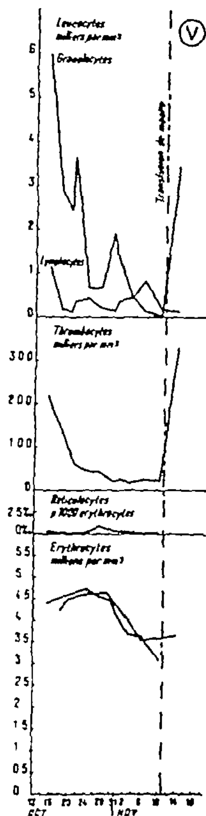


FIG. 1
Évolution des taux des différentes cellules dans le sang périphérique de V
(Rev. Fr. Et. Clin. Biol. 1959, mars)

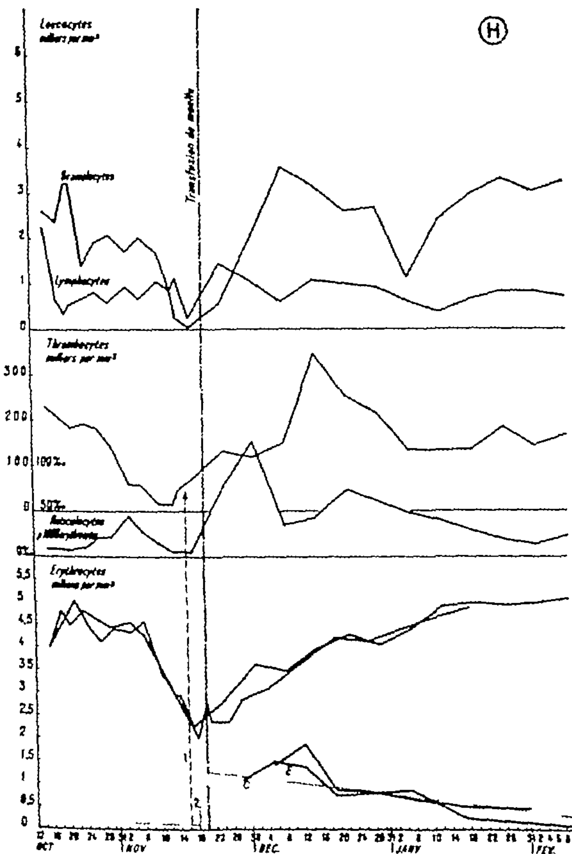


FIG. 2.

Évolution des taux des différentes cellules dans le sang périphérique de H. Courbes E et C : évolution de la population érythrocytaire produite par la moelle transfusée (E est la courbe de cette population établie grâce à l'antigène E présent sur les hématies du receveur. C est la courbe de cette population établie grâce à l'antigène C présent sur les hématies produites par la moelle transfusée). La flèche 1 indique une transfusion de plaquettes. Le pointillé 2 indique une transfusion de sang.

(*Rev. Fr. Ét. Clin. Biol.*, 1959, mars.)

La diminution secondaire du nombre des érythrocytes produits par le greffon ne semble pas liée à une hyperhémolyse et l'on a mentionné plus haut que les tests de Coombs pratiqués à cette période furent négatifs. La reprise de la production érythrocytaire propre des sujets greffés indique que des cellules souches ont échappé à la mort par l'irradiation (*) et l'on sait par des observations enregistrées chez l'animal que ce nombre peut être infime. La négativité du test de Coombs, l'absence d'anémie au moment du changement de la production cellulaire suggèrent que la diminution de la population cellulaire du greffon n'est pas due à l'hémolyse rapide de celle-ci par réapparition des fonctions immunologiques du receveur mais plutôt à une diminution de sa production. On peut soupçonner à l'origine de celle-ci un épuisement des cellules souches administrées ou l'état d'infériorité du greffon dans la compétition qui s'établit entre ses cellules et celles du receveur qui ont échappé à la mort par irradiation.

Si le rôle thérapeutique de la greffe apparaît certain si on peut lui attribuer la genèse des cellules sanguines pendant un mois si l'on peut de façon non moins certaine affirmer que la population érythrocytaire actuellement produite appartient à la propre moelle des patients peut-on prévoir pour autant un bon pronostic vital ? Nous ne discuterons pas ici l'effet de l'irradiation aux doses reçues par chacun des sujets sur la durée de vie, ainsi que les éventuelles complications (lésions des organes des sens, des organes génitaux cancers ou leucémies radio-induites) qui seront étudiés ailleurs [29] pour n'envisager que le problème du syndrome secondaire après greffe de moelle homologue. Si son mécanisme semble complexe et pouvoir être multiple chez les petits animaux (rats souris) qui en ont permis une étude approfondie [illegible] son existence et sa gravité chez l'homme irradié et restauré par de la moelle homologue ne peuvent être considérées comme certaines et cela peut être espéré pour trois raisons au moins *a*) ce syndrome ne semble pas avoir la même fréquence dans les diverses espèces animales il paraît rare chez le lapin [44] et le singe animaux dont la moelle comme celle de l'homme renferme beaucoup moins de « cellules lymphoïdes » que celle des rongeurs *b*) ce syndrome secondaire semble induit dans la majorité des cas par une immunisation du greffon contre l'hôte on peut donc espérer son absence lorsque la production cellulaire de l'hôte remplace celle du greffon une réserve doit cependant être faite dans le cas de nos patients chez lesquels la population leucocytaire ne peut être identifiée il est en effet probable mais non certain, que la production leucocytaire ait la même origine que celle des hématies des souris irra

(*) Dans l'article réf 29 on donnera les raisons indiquant que l'irradiation n'a pas été rigoureusement homogène Il est donc possible que certaines cellules aient reçu une dose moindre que la majorité des cellules corporelles

diées et restaurées par de la moelle non compatible ayant été observées qui produisaient une lignée cellulaire du donneur et une autre d'elles-mêmes c) on peut enfin espérer que, si le syndrome apparaît chez des humains irradiés et restaurés par de la moelle homologue, il puisse être plus ou moins accessible à la thérapeutique, en particulier dans certains de ses aspects physiopathologiques (*)

2) *Patients leucémiques*

Après avoir assisté à la restauration médullaire et sanguine et à la survie de ces sujets irradiés à de telles doses, nous avons pensé être moralement autorisés à irradier des patients leucémiques de façon quantitativement semblable.

Nous avons actuellement appliqué la méthode comportant une irradiation gamma totale et homogène à dose élevée (entre 850 et 900 rad) (**) suivie de transfusion de moelle homologue à trois patients atteints de leucose aiguë lymphoblastique. Ce traitement a été réalisé, pour les trois pendant une rémission.

La raison du choix de ce moment réside dans les résultats obtenus par cette méthode chez des souris porteuses de leucémies spontanées résultats d'autant plus décevants que la leucémie est plus hyperplasique et d'autant moins défavorables que le nombre de cellules leucémiques est plus faible [24, 25].

Nous avons par ailleurs observé [25, 44] dans le cas d'une leucémie DBA2 greffée sur des hybrides F_1 (DBA2 × C57BL6) et traitée par une irradiation à la DL 100/30 suivie de greffe de cellules hématopoïétiques que les cellules médullaires de C57BL6 adultes sont notablement plus efficaces que les cellules médullaires de F_1 adultes ou que les cellules myéloïdes embryonnaires de C57BL6. Il est tentant d'expliquer cet effet par une réaction immunitaire contre les cellules leucémiques DBA2 des cellules de souris C57BL6 adultes (homologues à l'égard de la leucémie). Cet effet immunitaire antileucémique n'est observé que si le nombre des cellules leucémiques est faible.

Les résultats de cette expérience nous ont donc maintenus dans l'idée de traiter les patients en période de rémission (**) et nous ont conduits à utiliser la moelle homologue de donneurs adultes.

La dose d'irradiation a été fractionnée en deux. Chaque fraction a été administrée en une dizaine d'heures.

(*) Signalons que nos sujets ne présentent pas d'hypogammaglobulinémie accentuée qui a été considérée comme pouvant jouer un rôle important dans le déterminisme des accidents secondaires.

(**) Ces malades ont été irradiés en collaboration avec Mme Dutreix, Mme Schweisguth, MM. Lalane, Pierquin et Denoix et suivis en collaboration avec Mme Larrieu et MM. Jean Bernard, Schwarzenberg, Van Putten, Schwarzmann, Céoard et Rapin.

Pour la première malade (enfant de 13 ans en quatrième rémission apparemment complète) une *première transfusion de moelle osseuse* d'un donneur du même sexe a été faite le lendemain et le surlendemain de la seconde irradiation (14 milliards de cellules nucléées soit 450 cm³ de liquide médullaire). Sept jours après cette injection, la moelle osseuse étant désertique, la cytopénie sanguine extrême et l'état général très gravement atteint, une transfusion de moelle d'un autre donneur du même sexe a été faite (20 milliards de cellules nucléées).

Pour les patients 2 et 3, âgés de 4 ans, une première transfusion de moelle osseuse (7 milliards de cellules nucléées) a été faite 6 jours seulement après l'irradiation. Devant la persistance de l'aplasie, une seconde transfusion de moelle osseuse du même donneur a été faite respectivement 4 jours après pour le patient n° 2 (4 milliards de cellules nucléées) et 6 jours après pour le patient n° 3 (6,5 milliards de cellules nucléées).

Le troisième patient est décédé en aplasie totale d'une fausse route, l'autopsie ayant montré la présence d'une grande quantité de vomissement dans les bronches.

Chez les deux autres, des cellules ont réapparu dans le sang environ 15 jours après l'irradiation.

L'étude de l'état général et de la courbe de température permet de distinguer trois périodes après l'irradiation : une première d'aplasie tolérée sans fièvre (*) ; une seconde d'aplasie décompensée avec une fièvre oscillante et diverses complications (nécroses, infections, hémorragies) ; une troisième de calme clinique annoncé par la chute de la fièvre et la réapparition d'un bon état général qui coïncident avec la réapparition des granulocytes dans le sang.

La première malade est actuellement en vie 70 jours après l'irradiation. Son hémogramme et son myélogramme sont normaux. Elle présente cependant actuellement un syndrome dont le début a été rapide et qui comporte des troubles digestifs (anorexie, diarrhée, insuffisance de digestion des aliments), de la toux, une fonte musculaire (qui fait actuellement l'objet d'un traitement symptomatique) et diverses lésions cutanées infectieuses.

La seconde malade, en vie actuellement 40 jours après l'irradiation, a un état hématologique et général satisfaisant. Elle présente cependant des plaques cutanées du type streptococcique en traitement par la pénicilline et un début de syndrome digestif semblable à celui de la précédente.

(*) On doit cependant signaler l'existence dans cette période des importants signes digestifs observés pendant l'irradiation (vomissements) et les jours suivants (diarrhée, vomissements).

Les deux sujets en vie ont en circulation dans le sang des hématies du phénotype du donneur de moelle mais en quantité insuffisante pour expliquer entièrement l'ascension du taux de leurs hématies.

La présentation de ces données préliminaires sous un aspect très résumé ne saurait être considérée comme une publication mais comme partie du commentaire de notre rapport.

III — Discussion Bilan des acquisitions

Nous nous limiterons dans le présent chapitre à dégager et préciser d'une part les notions acquises, d'autre part les questions et problèmes posés et encore sans réponse ni solution

Parmi les acquisitions valables les principales sont les suivantes *a)* il est possible d'injecter par voie veineuse, sans accident grave, des cellules myéloïdes embryonnaires ou de la moelle osseuse prélevée chez des adultes une filtration n'est pas nécessaire l'héparine peut être u… lisée comme anticoagulant *b)* la moelle isologue ainsi administ… voie veineuse semble se comporter comme chez l'animal, singuliè… en ce qui concerne la maturation des érythroblastes *c)* la gref… moelle homologue ne semble pas possible si le receveur ne reçoit … traitement conditionnant ou s'il est traité par les stéroïdes co… surrénaliens, même à dose très élevée ou par une irradiation à … égale ou inférieure à 500 rad *d)* en cas d'échec de prise de la gr… on observe le passage en nombre notable de plasmocytes et autres … lules hyperbasophiles *e)* les humains peuvent être soumis à une ir… diation transcutanée totale d'environ 850 rem (rayons gamma, neutro… sans présenter obligatoirement de radio-lésions irréversibles s'ils reç… vent après l'irradiation de la moelle osseuse par voie veineuse *f)* un… telle irradiation est suivie d'une aplasie myéloïde et lymphoïde total… tolérée grâce au traitement symptomatique pendant quelques jours *g)* des sujets ainsi irradiés et aplasiques peuvent survivre si la restaura… tion des organes hématopoïétiques et du sang est survenue avant la phase de décompensation de la cytopénie sanguine *h)* cette restaura… tion peut être liée à la greffe de la moelle *i)* la dose d'irradiation de 850 rem est suffisante pour conditionner l'hôte à l'égard d'une greffe de moelle transitoire *j)* le nombre de 10^{10} cellules myéloïdes nucléées homologues semble suffisant chez un adulte pour assurer une restaura… tion des organes hématopoïétiques et du sang *k)* la greffe de moelle semble possible quand elle est injectée 25 jours après l'irradiation

Parmi les questions encore sans réponse, on peut inscrire les suivantes Peut-on administrer pour restaurer un sujet ainsi irradié, la moelle de plusieurs donneurs homologues simultanément ou successi…

vement ? Une irradiation unique est-elle préférable à une irradiation fractionnée, un débit lent à un débit rapide[14] ? Quels sont les meilleurs fractionnements, le meilleur débit ? Les divers rayonnements pourvu qu'ils soient suffisamment pénétrants sont-ils identiquement efficaces pour le conditionnement du receveur à l'égard de la greffe de moelle homologue : rayons gamma, rayons X émis sous haute énergie (par le bêtatron par exemple), neutrons, mélange de radiations ? Ou l'une des variétés de rayonnement est-elle supérieure aux autres ? Existe-t-il un moment optimum après l'irradiation pour l'injection de la moelle restauratrice ? Peut-elle se comporter différemment, en particulier en ce qui concerne respectivement les phénomènes de maturation et de division selon qu'elle est administrée tôt après l'irradiation ou assez tard ? Existe-t-il un nombre optimum de cellules à administrer ? Est-il préférable que donneur et receveur soient du même sexe, en d'autres termes, l'histocompatibilité liée au sexe[3, 41] doit-elle être prise en considération ? Doit-on redouter chez un malade ayant reçu des transfusions de sang homologue avant l'irradiation, un éventuel échec de la greffe lié à une immunisation[8] ? Une greffe homologue définitive est-elle possible chez l'homme ? Quelles sont les conditions qui détermineraient le caractère définitif ou transitoire d'une greffe de moelle homologue ? Sont-elles liées à la dose d'irradiation ou aux cellules administrées (nombre, parenté génétique avec le receveur) ? La maladie homologue peut-elle exister chez l'homme après greffe de moelle homologue ? Son éventualité est-elle à redouter en cas de greffe transitoire ? Est-elle obligatoire ou seulement possible en cas de greffe éventuellement définitive ? Serait-elle sensible aux thérapeutiques symptomatiques, aux stéroïdes corticosurrénaux ? La durée de vie des sujets ainsi irradiés et restaurés sera-t-elle plus courte que la durée de vie des sujets normaux (en raison de séquelles ou de complications secondaires) ? L'éventuel raccourcissement dépendra-t-il du caractère définitif ou transitoire de la greffe ? Serait-il évité par l'emploi de cellules myéloïdes embryonnaires ? Est-il même possible de restaurer des humains avec des cellules myéloïdes embryonnaires ?

Ce ne sont là que les principales questions posées, de nombreuses autres leur sont liées et la solution de l'une d'entre elles en entraînerait d'autres encore.

Avant que ces solutions ne puissent être apportées, on peut cependant : *a*) conclure que la transfusion de moelle osseuse est désormais inscrite parmi les moyens thérapeutiques dont on dispose pour secourir les accidents par irradiation à dose létale ; *b*) utiliser les données nouvelles pour poursuivre les recherches concernant l'application de l'irradiation totale à haute dose suivie de transfusion de moelle osseuse aux recherches thérapeutiques de certaines variétés de leucémie, envisager

des recherches et d'autres applications médicales éventuelles singulièrement la transplantation homologue de tissus ou d'organes[4, 9, 51]

RESUME.

L'auteur décrit la technique du prélèvement de la moelle osseuse chez le donneur et de l'injection chez le receveur. Il aborde les principaux problèmes posés par le conditionnement de celui-ci en cas de transfusion de moelle homologue (par l'irradiation transcutanée), la surveillance et les méthodes utilisables pour étudier la prise de greffe.

Il décrit ses premiers essais qui furent des échecs chez des patients leucémiques non conditionnés traités par les corticostéroïdes ou irradiés à des doses insuffisantes.

Il rapporte un cas de transfusion de moelle entre jumeaux.

Il discute les résultats obtenus chez cinq sujets victimes d'une irradiation accidentelle à très haute dose [résultats qui sont actuellement en cours de publication (*Rev. Fr. Et. Clin. Biol.*, mars 1959)].

Il cite les premiers résultats observés chez trois patients atteints de leucémie lymphoblastique et soumis, en période de rémission complète, à une irradiation à dose très élevée suivie de transfusion de moelle osseuse. Les raisons du choix de ce moment (période de rémission) pour appliquer cet essai de traitement et du choix de la moelle homologue adulte sont discutées à la lumière de données expérimentales personnelles qui sont brièvement rappelées.

SUMMARY

The author describes a technique of collecting bone marrow from the donor and injecting it to the host.

Some problems are investigated such as conditionnement of the host through transcutaneous irradiation for transfusion of homologous marrow, the survey and the methods available to check the success of the graft.

A case of bone marrow transfusion between twins is reported.

Are also reported cases of homologous transfusions of marrow which failed, in patients who were not conditioned, treated by corticoids or irradiated at insufficient doses.

The results so far obtained in 5 subjects submitted accidentally to massive doses of radiations are discussed. (These cases are being published in a paper of the *Rev. Fr. Et. Clin. Biol.*, March 1959).

Three patients with lymphoblastic leukemia were submitted in a stage of complete remission to high dose gamma irradiation followed by transfusion of homologous bone marrow.

The reason of selecting patients at this stage of the disease and of choosing adult homologous marrow for the graft are briefly analysed with reference to personal experimental data which are exposed.

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DISCUSSION

MERRILL. I would like to congratulate Dr Mathé on his work. Knowing the problems of caring for patients who have been totally irradiated I am in a position to understand the immense difficulties which he has surmounted.

Dr Murray and I have studied three patients in whom whole body radiation was given in preparation for a kidney homograft. The first received 600 r with a 2 MEV apparatus. She was given pooled marrow from male donors. There was no real evidence of cell production in the peripheral blood although at autopsy the bone marrow was hyperplastic. We could not be sure however whether donor or recipient cells had repopulated the marrow. The second case received 700 r and marrow from a single donor, his mother. Again there was no evidence of take and at autopsy the marrow was aplastic. The third case reported earlier was the twin. He was given 250 r and then 200 r from a 250 Kv apparatus. No marrow was given. After a critical period of agranulocytosis his own marrow resumed function.

In all these patients the problem of infection—with their own and environmental flora—was critical. Since we can care for patients better than the experimental animal this appears even more critical in work on whole body irradiation in the latter and I would reemphasize Dr Van Bekkum's point about the role of infection in « homologous disease ».

From our experience and from Ferrebee's it would seem that for successful marrow take in the human one should go well above the 600 r range being careful to avoid the range at which acute radiation disease will occur. I am therefore surprised that in Mathé's patient

receiving 480 r the marrow graft was successful Perhaps I have misunderstood. Did this patient actually get a marrow infusion?

MATHÉ We don t have any evidence of the take of transfused bone marrow in patients with a small dose (for example 600 r [200 Kv] for one leukaemic patient and 500 rad [betatron] for another one)

MERRILL Thank you, this fits much better

WOODRUFF I should like to add my congratulations to Dr Mathé not only on his scientific skill but also on his courage and to mention briefly one case McWhirter Newall and I have treated in Edinburgh. Fearing secondary disease we decided to use exclusively foetal homologous liver for replacements and limitations of availability dictated the use of younger foetuses than we would have wished. The patient, who was suffering from disseminated seminoma of the testis was given 500 rads total body irradiation on a 4 MeV linear accelerator followed by intravenous injection of 5·6 × 10⁹ cells prepared from the whole livers of three human foetuses of 12 16 weeks gestation The patient lived for 17 days after irradiation, during which time the secondaries in his lungs became demonstrably smaller on radiological examination. Lymphocytes polymorphs and platelets disappeared completely from his peripheral blood but subsequently mononucleate cells reappeared in the blood stream and reached a count of 1 600/cu mm The nature of these cells is unknown, but the patient certainly showed neither polymorphs nor platelets in his blood. Various explanations may be suggested but the results at least raise the possibility that liver from foetuses as immature as these we used may be incapable of producing adequate replacements of all blood cell types

MATHÉ We obtained «embryonic» myeloid cells from the spleen and the liver of a premature child (foetal age 5 months) whose death was not due to infectious causes. As I said in my presentation we injected these cells intravenously into one of the accidentally irradiated subjects without any result but the period of observation before injecting adult bone marrow was too short for us to conclude it had not been efficient.

VOISIN May I make a suggestion, or rather ask a question? Would it not be of help to make blood transfusions into the irradiated patients from the donor of the bone marrow during the « stormy » period which follows the blood marrow transfusion?

MATHÉ. Do I understand that you expect a reaction from the leucocytes of the transfused blood against the antigens of the grafted bone marrow if the blood and marrow donors are different?

VOISIN. Yes. Such a procedure would have two advantages. First to help the patient over the period when the transfused bone marrow is expected to become active and second using the same donor would avoid the possible harmful consequences in these enfeebled patients of a supplementary immunological fight between the immunologically competent cells of bone marrow donor origin and the blood cells of the blood transfusion donor.

MATHÉ. We don't have any data yet to make us think this effect would occur. And I must tell you that it would be asking too much of the marrow donor to give blood subsequently.

I am more afraid of the eventual effect of blood transfusions received by the patients *before* irradiation. There are some experimental data which suggest that irradiation inhibits an immune response to an antigen given after the irradiation but does not inhibit if the antigen has been given before irradiation.

The absence of vascularization as a cause of the destruction of homografts Experiments to improve the results

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In transplantation research work I endeavour to find out a way to extend the survival time of homografts a kind of adaptation in the sense of Woodruff or of transformation which is suitable for the surgeon With this procedure in mind we studied the development of auto- iso- syngenisio- homo- and heterografts of liver and spleen of rats and grafts of thyroid and kidney of rabbits

By theoretical considerations we came to the conclusion that the lack of blood vessels during the first few days is of great importance for the success of all grafts, both autografts and heterografts. During this period they become heavily damaged by a lack of food substances and oxygen and by accumulated waste products. The thicker the implanted tissue pieces, the bigger the central cell layers will be which become irreversibly injured (Fig 1) For this reason all grafts should be arranged so that, in absence of a functioning blood circulatory system diffusion from the graft bed into the graft itself can supply its basic minimum demands while freeing it from metabolism products. For this reason we work with very thin grafts sliced by a razor [10]

In addition to this non-specific damage affecting autografts as well as homo- and heterografts the homo- and heterografts are also afflicted by specific injuries caused by their foreignness to the receiver animal's body Almost all researchers in this field following the pioneer work of Leo Loeb [11] agree that genetic factors are involved in graft compatibilities. It is a general rule that grafts will only take on a foreign host if they find there their own histocompatibility genes

The immunogenetic conception is wide spread with respect to the mechanism by which these particular genes bring about or prevent the taking of the graft. We do not agree with this conception in that respect that those homografts which we studied should be destroyed exclusively or mainly by the antibodies which may be elicited by them. It may be that antibodies are auxiliary in this respect. Our histological study of the development of grafts convinced us that the most striking



FIG 1 — Autograft of a complete rabbit thyroid lobe not sectioned by a razor implanted subcutaneously 22 days ago. Though it is an autograft, the broad inner layers of the thyroid are composed of fibrous tissue, among which some atrophic epithelial cells, multinuclear giant cells, lymphocytes and plasma cells are scattered. The epithelial parenchyma in this part was irreversibly injured during the long period until granulation tissue with blood vessels could reach these cell-layers now because the graft is 2 or 3 mm thick. At the border of the graft several rows of thyroid follicles have survived: they were able to survive because they were sufficiently nourished and freed of waste products by diffusion from the graft bed nearby.

factor determining the fate of grafts is the directly opposite behaviour of blood vessels around and in autograft in comparison to homografts. Our histological investigations cover[illegible] rted fr[illegible] rst day after transplantation until three to fiv[illegible] ate

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FIG. 2 — Autograft of rabbit thyroid 22 days old in the omentum (razor-section). In general the graft consists of healthy thyroid tissue. Some parts are still in a stage of recovery

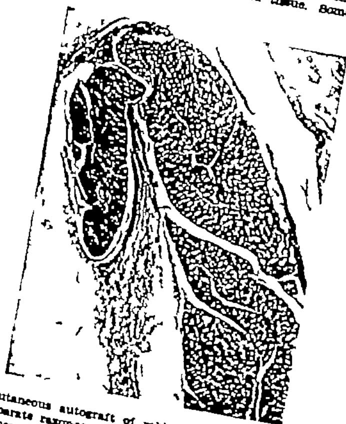


FIG. 3 — Subcutaneous autograft of rabbit thyroid 6 weeks old. Originally it was composed of separate razor-sections, which grew together by granulation tissue. The vessels became permanent. In this manner the graft received a sufficient blood supply. In the bunch of connective tissue vessels run to and from the graft. The graft consists of a great number of follicles with high epithelial cells which generally are colloid free

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factor determining the fate of grafts is the directly opposite behaviour of blood vessels around and in autografts in comparison to homografts. Our histological investigations cover a period from the first day after transplantation until three to five months later.

During these studies we observed that in the long run only the autografts received a sufficient quantity of blood. In these latter cases the vessels of the graft bed wound healing tissue turned into permanent



FIG. 2 — Autograft of rabbit thyroid 22 days old in the omentum (razor-section) In general the graft consists of healthy thyroid tissue. Some parts are still in a stage of recovery

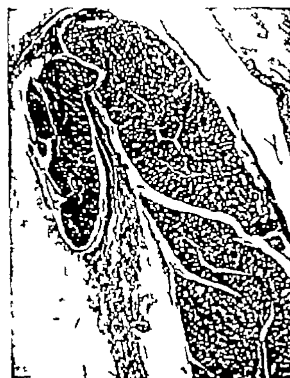


FIG. 3 — Subcutaneous autograft of rabbit thyroid 6 weeks old. Originally it was composed of separate razor-sections, which grew together by granulation tissue. The vessels became permanent. In this manner the graft received a sufficient blood supply. In the bunch of connective tissue vessels run to and from the graft. The graft consists of a great number of follicles with high epithelial cells which generally are colloid free.

vessels of the graft (Fig 2 3) Homografts however enjoy only a sham flourishing period during the first 18 days or so that is to say just as long as they are imbedded in pushing wound healing tissue (Fig 4, 5) As soon as the wound in the subcutaneous layer containing the graft is healed that is to say as soon as the wound healing tissue changes into fibrous granulation tissue the condition of the graft becomes critical (Fig 6). The epithelial parenchyma fades away the



FIG. 4. — Rabbit thyroid homograft as a razor-section subcutaneously transplanted 7 days ago. It is embedded in soft wound healing tissue. Some vessels are extended and filled with erythrocytes (in the picture appearing very dark) Many follicles have remained intact, with or without colloid. For some time the parenchyma cells may profit by the vessels of the granulation tissue even in case the blood is stagnant, because there is a supply of oxygen in it. Lymphocytes are at the border and are about to infiltrate the homograft.

remainder turns into sclerotic tissue over which lymphocytes plasma cells and some other cell types may be scattered (Fig 7).

Many investigators consider lymphocytes and plasma cells as the typical allergy cells. Indeed in recent years much evidence has been produced substantiating the assumption that plasma cells are the main producers of antibodies. However the proteins they build up are not always antibodies [2, 3, 4, 12, 19] We should keep in mind the many plasma cells in chronic salpingitis for instance

According to Marchand [14] Borst [4] Mallory [15] etc. the presence of lymphocytes and plasma cells combined to some extent with eosinophilic and basophilic leucocytes, signifies an unspecific chronic inflammation. In the 1920 s it was believed that irritants originating from degenerating and dying cells would attract lymphocytes and plasma cells. According to other scientists perhaps the change in pH produced by waste products, especially *lactic acid* should attract lymphocytes and plasma cells. Menkin [18] presented his leukotaxin. However expe



FIG. 8 — Homograft of rabbit thyroid 18 days old, implanted subcutaneously as a razor-section. It is in a rather good condition, not worse than an autograft of the same age. This is true because it is still profiting of the vascularisation by the wound healing tissue But this help is only temporary

riments failed to show lymphocytes to be sensitive to chemotaxis [8] According to the older pathologists such as Marchand [14] Mallory [15], etc. the task of these infiltrating cells should be that of freeing the afflicted tissue of these irritants by absorbing them. Today however the absorbing function of lymphocytes does not seem probable, because they have no well developed Golgi apparatus [8] Since they appear in autografts too we would suggest the theory that they may help in repairing the afflicted tissue. There are hints that the short lived lymphocytes after breaking down set metabolites and energy free, both of which are useful for *cell synthesis*. *Plasma cells*, on the other hand are generally believed to build up and to release protein [8]

We feel that the development of *homografts of normal tissues which* we studied can be easily understood with our point of view in mind. Their development seems promising during the first 14 to 18 days or so, that is to say as long as the grafts are embedded in hyperaemic wound healing tissue from which they receive their nourishment mainly by diffusion. The critical point is reached when the wound healing tissue changes into fibrous granulation or scar tissue with occlusion of most of



FIG. 6 — Homograft of rabbit thyroid 31 days old, implanted subcutaneously as a razor-section. There are still many intact follicles. But in other ones the epithelial cells are fading. Piles of lymphocytes have interspersed the tissue

its blood vessels. At this time the grafts starve and suffocate. Their parenchyma cells become atrophic, degenerate and die. The typical cells of chronic inflammation, mainly lymphocytes and plasma cells, are involved in the repair process. The remainder of the homograft becomes sclerotic.

In order to avoid this fatal result in homografts we carried on two groups of experiments. In the first group, using rabbit thyroid homografts, we aimed at compensating the faulty blood supply at least partially. To this end we brought about an exudation of blood plasma out of the vessels of the host rabbit hoping that such an overflow of the graft bed with nutrients would benefit the starving homograft. Of course this

procedure did not give the tissue the oxygen it required. The plasma exsudation was caused by daily injections of 1 ml of histamine-hydrochloride begun on the day following implantation. The injections were made subcutaneously either in the pockets containing the grafts or at various places on the body. We continued this treatment in some of the rabbits up to ten weeks. There was an even greater number of



FIG. 7 — Subcutaneous homograft of rabbit thyroid 8 weeks ago implanted as a razor-section. It is surrounded by a dense capsule. The graft itself has become sclerotic. There are only a few lymphocytes. The sclerotic tissue originates partly from the granulation tissue of the host, partly it derives from the scanty stroma of the thyroid graft itself.

control animals with grafts of the same type but without injections of histamine hydrochloride.[19]

In fact in 10 out of 12 rabbits the usual sclerosis of the thyroid homografts did not occur. The grafts retained vitality in varying degrees. There were no well formed follicles left. Yet the epithelial cells of the histamine-treated homografts differed from those in non treated animals. They did not fade away as did the latter. On the contrary they showed great variations in size of the cells as well as size of the nuclei (Fig. 8). At times there were very large and eosinophilic nucleoli in them though the nuclear membrane had a shrunken appearance. We found a good number of mitoses, many of them with abnor

mal features consisting of chromosome aberrations, unequal chromosome distribution and hyper and hypoploidy On two occasions parts of the histamine-treated graft had adenoma like structures One of the grafts had developed into a benign fibro-epithelial tumour Partly it scarred with opulent collagenous fibres. Partly however it exhibited growth centers consisting of groups of fibroblasts and epithelial cells with a surprising number of mitoses, a high percentage of which were hyperploid and pathologically disturbed in different ways



FIG. 6. — Rabbit thyroid subcutaneous homograft treated with subcutaneous injections of histamine hydrochloride for 5 weeks (razor-section) The capsule encloses the remainders of the graft. The graft differs much both from autografts and from untreated homografts. It does not consist of collagenous scar tissue but is composed of connective tissue and epithelial cells mixed up without an organotypical arrangement. There is only a certain degree of fibre formation Lot of multi nuclear giant cells are present.

The results of this group of experiments do not support the assumption that homografts are destroyed by antibodies. If histamine has any significance in the immunogenetic conception however then the direct opposite influence is to be expected that is to say histamine would destroy but not promote homografts. In fact this method of extending the survival of homografts is useless for the surgeon because it is dangerous however it proves once again that homografts can be protected from destruction Moreover we feel it supports our hypothesis that the

lacking blood vessels are the true cause for the fatal development of homografts.

In our second series of experiments we endeavoured to save kidney homografts from destruction in that we tried to transform their own genes especially their histocompatibility genes into genes identical with those of the receiver animal. Assuming that we are successful in this rather daring undertaking the homograft, although originating from a foreign individual would find compatibility genes identical to its own at its new location. This is as we all know very well the most elementary requirement for its taking on the new host.

We applied the method known to be effective in transforming bacteria from one type into another[3, 4] Thus, we treated the grafts with highly polymerized DNA from the receiver animal in various ways. We proceeded in the following manner

We began by removing by biopsy the left kidney of the future host rabbit. Deoxyribonucleic acid (DNA) was then prepared of it by my assistant, Dr Stein von Kamienski With slight modifications he followed the method of Gulland and his coworkers This method takes special care to avoid depolymerization The resulting product was very viscous. It contained 6 to 7 % RNA and was free from protein. Five to nine gr of fresh kidney yielded 10 to 19 mg DNA. Until needed the DNA was dissolved in 0 14 m NaCl plus 0,01 m Na-Citrate. This solution was then frozen and refrigerated at — 8 C.

The homografts were taken from the kidney of a non related rabbit. We cut the organ into sections of some hundreds μ by a razor The trials themselves as well as the controls were implanted into different subcutaneous pockets in the flank of the previously nephrectomized rabbit The trials were treated with DNA in various different ways. The controls were either not treated at all or only with the solvent i.e. a Tyrode solution with sodium citrate and the rabbit's own blood serum taken from its ear vein.

The graft trials were treated with DNA as follows

They were bathed for 15 minutes in 2 ml of Tyrode solution containing 0 5 mg DNA plus 0,1 ml sodium citrate 12 % and 1 ml of the rabbit's own blood serum. One ml of the same solution was injected into the same pocket. This was repeated 5 to 20 times

Further kidney razor sections were grown as tissue cultures swimming in a lactalbumin-yeast nutrient medium with or without DNA, depending on whether they were trials or controls. Seven days later both were implanted in separate pockets of the rabbit whose kidney provided the DNA.

Then we pressed DNA solution (or the solvent without DNA) into freshly cut kidney sections. This was done by releasing the vacuum existing in an exsiccator containing the sections in the solution. They were implanted in a pocket of the DNA-donor rabbit.

Finally we filled the pockets of the unilaterally nephrectomized rabbit with a tissue culture medium consisting of 1 ml hen's blood plasma plus 1 ml of the rabbit's own blood serum plus 2 ml chicken embryo juice including for the trial grafts 2 mg DNA with 0,1 ml sodium citrate 12 %. Several sections were put into each pocket. Clotting occurred almost immediately in the control pockets without DNA. In the pockets with DNA clotting did not occur. We sewed up the pocket containing the immersed sections after 15 minutes.

After 5 to 8 weeks of implantation we removed all homografts and studied paraffin sections of them.

Up to now we have not achieved any real improvement in homografts treated with DNA over those not treated with DNA. However there are a few minor peculiarities in the DNA treated grafts which encourage us to continue the experiments.

As far as we know some researchers who applied the transforming method with DNA to tissues of vertebrates failed to succeed as we did. Medawar [17] tried to achieve active tolerance for skin homografts by injecting DNA into baby mice over a rather long period beginning at birth. The DNA was prepared of tissues of the same mouse strain which will supply the skin homografts later on. There was no positive result in 25 trials.

Benoit, Leroy, Vendrely and Vendrely [1] tried to transform Pekin ducklings into ducks of the Khaki Campbell type by injecting DNA of Khaki Campbell duck cells into male and female Pekin ducklings beginning 8 days after hatching. The majority of the animals treated thusly as well as their offspring in the first generation exhibited some features occurring in Khaki ducks. Unfortunately however according to Greenwood these features sometimes occur spontaneously in Pekin ducks too. Greenwood in *Nature* refers to the paper of the French authors. He stresses the great importance these results would have should they be substantiated by further experiments. He concludes by quoting the authors themselves: « Aujourd'hui notre meilleure conclusion sera que nous n'avons aucune conclusion à vous presenter » (« Today our best conclusion will be that we don't have any conclusion to offer you. »)

I myself am in the same situation as to the homograft experiments with DNA which I have just presented you. However I feel it would be worth while to continue with this type of experimentation.

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DISCUSSION

MEDAWAR I feel that Dr Knake's chief point—a very important one—is that we do not know the immediate cause of the death of the cells in homografts—or at all events in solid homografts What most of us believe is that immunological incompatibility underlies the destruction of homografts though the final pathway by which it takes effect may sometimes be a vascular change. The pathological changes that occur in a second set skin homograft are indeed mainly ischaemic but the failure of vascularization has an immunological origin. Otherwise why should there be a difference between first-set and second-set homografts ? Is there not a danger that Dr Knake's treatments are purely symptomatic ?

KNAKE That is just what I want them to be ! I want to help the homograft to adapt itself to transform My purposes are purely practical ones. I suppose that the histocompatibility genes influence the vessels of the graft and I hope to support the graft to make good the absence of blood vessels.

HYATT Have you any suggestion for promoting the survival of large homografts ? How thick can your homografts be ?

KNAKE You ask how big a distance can be bridged by diffusion ? Only about 300 or 400 microns. The best grafts in my experience are the most transparent and these are about 300 or 400 microns thick

KLEIN With some tumour cells (which are of course very different from yours) when one mixes a very small percentage of isologous cells with a high proportion of homologous cells the isologous cells actually survive better than when they are alone Have you tried putting a small autograft in a larger homograft to see if it survives ?

KNAKE I have not tried

MEDAWAR I have and it does.

VOISIN I would like to make a brief and accessory comment concerning lymphocytes and mononuclear infiltration in thyroid autografts. The fact that mononuclear cell infiltrations were observed in thyroid autografts was taken as an evidence that this infiltration had nothing to do with an immunological reaction I would suggest that it might actually be a sign of an immunological reaction since thyroid tissue is

one of the very few able to induce auto-sensitization. In this case, in particular the experimental manipulation allowed substances from the thyroid to reach the immunological centres which does not happen under normal physiological conditions. As a matter of fact, the picture that we saw of a thyroid autograft infiltrated with mononuclear cells resembled very closely the known cases of experimental « allergic » thyroiditis

Fate of skin grafts in irradiated rabbits treated with marrow from single and multiple donors ( )

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Two patients each of whom had lost a solitary kidney through trauma or surgery were recently treated with total body irradiation marrow transfusions and a subsequent kidney or skin graft In such instances the ideal protocol would have been total body irradiation in a known lethal range followed by sufficient quantity of immature hematopoietic cells from an individual who would subsequently become the kidney donor For reasons of impracticality or lack of pertinent data compromises related to marrow dosage and the source and timing of the subsequent kidney graft were inevitable These human experiences have suggested several animal experiments which might furnish the necessary information to aid in the care of similar future patients Some of these experiments are the basis of this report

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In general the study may be divided into three phases. First, the use of pooled marrow from each of several donors was tested to determine whether the total marrow dose might be increased. Secondly skin grafts from each of the marrow donors were observed on the single recipient. The ability of these animals to accept skin from each of the successful marrow donors has been described previously[8] Survival of marrow was assumed if the skin graft from the marrow donor survived. Thirdly the timing of an indifferent skin graft after marrow infusion was studied to determine whether or not a kidney graft to be successful required immediate transplantation. If it could be ascertained that successful grafting could occur several days after marrow infusion, the logistics of human kidney transplantation could be simplified

Materials and Methods

The experimental scheme has been described in detail in a previous article[8] The salient features are 1) The recipient animals were white, male New Zealand rabbits genetically unrelated weighing 2500-3000 grams 2) The irradiation dose was 1100 r delivered in divided doses of 600 r and 500 r twenty four hours apart The factors were 250 kv and 15 Ma machine, T 3 filter HVL 3.0 mm Cu field size 20 × 20 cm target object distance 50 cm The X ray dose was delivered at an average rate of 35 r per minute expressed as an air dose at the center of the animal. All animals were rotated three times during the course of treatment to secure uniform distribution 3) Antibiotics were administered to all animals except to some of the control group starting on the day prior to irradiation and continuing for a total of thirteen days. Penicillin (80,000 units) and streptomycin (0 1 gram) were used 4) The donor animals were of a different strain than the recipients in almost all instances. Either the Dutch or California strains were used because of their colored fur These animals were six to eight weeks old weanlings, but immunologically mature. 5) Marrow was obtained from the tibiae, femora and humeri, placed in Hank s solution at a pH of about 7.4 and passed through progressively smaller sized stainless steel screens to break up clumps When pooled marrow was being used all of the marrow from the five donors was collected together in a single beaker and processed (Fig 1). 6) The bone marrow was infused within an hour after the harvesting had been completed and within two to three hours of the administration of the total X ray dose. 7) Full thickness skin grafts were applied to the recipient animals under intravenous Nembutal anesthesia as soon as the marrow had been given These were fixed in position with corner sutures only dressings were unnecessary 8) Skin grafts were observed and their status recorded

daily Complete blood counts smears for female tagged neutrophils and weights were taken on each recipient animal before irradiation and at least twice a week after marrow infusion

Plan of Experiments — Single marrow recipients received an autograft a marrow donor homograft and another homograft from a non-marrow donor i.e. an « indifferent » homograft Each pooled marrow recipient received an autograft, a homograft from each of the marrow donors (five in number) and in most cases an indifferent graft All these grafts were placed on the day of transplantation. An additional indifferent homograft was placed at 21 30 days and at 150 days in some of the animals.

Controls — One study consisted of cross skin grafts between untreated animals of the New Zealand strain to determine the mean primary and « second set » rejection times. A second group of controls was irradiated in divided doses of 600 r and 500 r at 24 hour intervals. They received no bone marrow but were skin grafted with an autograft and a homograft A third control group was the same as the second except that they received antibiotics.

Criteria for Graft Survival and Rejection — The ultimate evidence for homograft survival was a luxurious growth of hair characteristic of donor The criteria for « acute » rejection of skin were definite cyanosis and edema These signs were always followed by full thickness necrosis with 24 48 hours. The majority of animals reported below demonstrate the acute type of skin homograft rejection. However a chronic type of graft rejection was frequently observed in irradiated animals. Not only was the survival of the graft prolonged but the rejection period itself frequently took 10 to 12 days for completion The graft slowly thickened became edematous and scaled on its surface and hair growth ceased and necrosis of the homograft eventually took place Hair growth on successful grafts began on shaved grafts in about two or three weeks If the skin had not been completely shaved at the time of grafting some grafts demonstrated uninterrupted hair growth as the homograft survived.

RESULTS.

Controls — In the first control group of eight animals the mean rejection time was 6.6 days with a standard deviation of 1 ~ days The mean second set rejection was 3.1 days with a standard deviation of .4

The second group of controls of 12 animals who received 600 r and 500 r no antibiotics and did receive skin homografts and autografts all died within 12 days. The third control group of 18 animals had three survivors beyond 12 days. In these three animals the *autografts* remained intact but the homografts were rejected at 22.7 days (± 5 8)

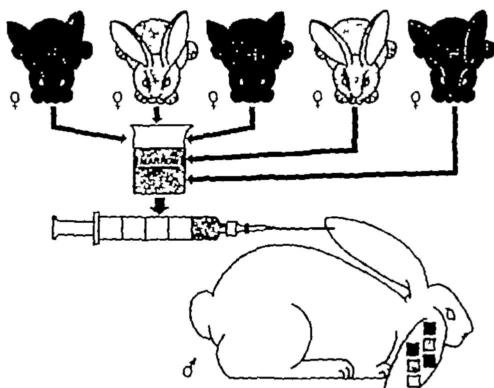


Fig. 1. — Pooled Marrow Experiment The marrow pooled from five female donors is injected intravenously into a single irradiated (600 r and 500 r) male recipient. Then skin is grafted from each of the marrow donors and in many cases from an indifferent donor A control autograft is also placed.

Experimental Material. — There were 40 animals in the pooled marrow donor series. Ten or 25 % died within the first week 8 on *the first day These were attributable to the irradiation, anesthesia, or* fat embolism from the marrow infusion Nine animals died at the end of the second week (22 %) 9 more animals died by the end of the third week (22 %) 3 died at the end of the fourth week (8 %) Thus at the end of four weeks, there were 9 survivors (22 %) Three animals died during the fifth week and two in the eighth week, and one in the tenth week. Three have survived beyond seventeen weeks (Table I)

All but 5 of the 21 animals who survived beyond two weeks had evidence of successful marrow graft as indicated by the presence of circulating neutrophils with the characteristic female chromatin marker Two of these five exceptions died on day fifteen but the other three

TABLE I — *Animal Mortality*

Days	1	2-7	8-14	15-21	22-28	29-35	50-56	63-70	*Still Alive*
No. of Animals (40)	8	2	9	9	3	3	2	1	3

have recovered weight and have survived apparently with their own marrow active and proliferating The successful marrow grafted animals dying during the second and third week had falling total leucocyte counts after being in a normal range with 2 6 % female labelled leucocytes present. Four animals dying in the fourth and fifth week and one dying in the eighth week had diarrhea increasing leucocyte count weight loss, and icteric serum Two animals still alive at 120 days demonstrate circulating female leucocytes.

There were 14 animals in the single donor marrow series four died on day zero The other ten survived beyond fourteen days, and five of them survived beyond twenty-eight days. One died at thirty-eight days and the other four lived beyond eighty five days

The results of skin grafts in the pooled marrow treated animals are indicated in Table II In animals surviving more than seven days

TABLE II — *Pooled Marrow and Skin Graft Survival*

No of Animals	*Week dying*	*Homograft*	*Indifferent*
1	2nd	—	0/1
7	3rd	28/34	3/3
3	4th	12 14	0/0
3	5th	7/15	0/1
1	7th	5/ 8	1/1
2 (with ♀ cells)	Alive at 17 Wks	4 10	0 4

Animals without Female Cells

No of Animals	*Week Dying*	*Homograft*	*Indifferent*
2	3rd	5 10	0 2
1	8th	0 8	0 1
1	10th	0 5	0 1
1	Alive at 17 Wk	0 8	0 1
		61 103	4 15

Numerator Grafts surviving
Denominator — Total number of grafts

27/30 control autografts, 148/150 donor homografts and 22/22 indifferent grafts were successful from a technical viewpoint. Because no significance can be given to graft survival under fourteen days, only those grafts surviving into the third week will be considered in the following analysis. Of the 21 animals surviving fourteen days or more, seventeen had two or more viable homografts with a total of sixty-one. Three animals accepted two grafts, five animals accepted three grafts, five accepted four, and four accepted five grafts. One animal accepted only one graft. The average number of successful grafts was 3.4 per animal. The three animals without female circulating cells rejected all grafts. In Table II the numbers of viable homografts are listed on a weekly basis.

All ten of the animals who received marrow from a single donor supported a skin homograft from the same donor to time of death of the animal. These are not listed on Table II.

TABLE III. — *Mean Survival of Homografts*

No. of Animals.	*Type of Graft*	*Mean Survival Time. (Days)*	*% Rejected*
3	Homograft on irradiated non-marrow infused host.	22.7 (± 5.7)	100 %
21	Homograft, Irradiated + Marrow (living beyond 14 days)		
	16 with ♀ cells	11.2 (± 3.3)	29 %
	3 without ♀ cells	23.0 (± 8.7)	100 %

( ) Two animals died without ♀ cells at fifteen days. 6/10 homografts still survived. Presumably by 23 days (± 8.7) all would have been rejected. These two animals have not been included in the above table.

The duration of survival of homografts from pooled marrow donors is indicated in a different manner in Table III. Note that the mean rejection time of homografts on successfully marrow treated hosts is shorter than on unsuccessfully treated rabbits. In this latter group the rejection time of 23.0 (± 8.7) is no different from the 22.7 (± 5.7) days survival for the non marrow infused irradiated group who happened to survive as their own marrow regenerated.

After pooled marrow 9/15 indifferent grafts died by eleven days. One rejected at fifteen days and one at thirty-four days. Four were alive at 15-21 days at which time the animals died. On single marrow

treated animals eight of fifteen indifferent grafts died by day eleven. Three were alive when the animals died at 15 22 days and one was still alive at day thirty-nine Three indifferent grafts however were rejected late at 5* 57 and 78 days.

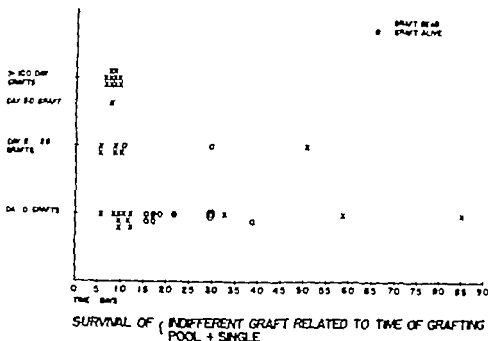


FIG. 2 — Survival of indifferent skin grafts plotted against day of grafting Note that grafts on day zero tend to survive somewhat longer than those applied later but 72 % died by day ten. Total numbers are not significant.

The survival of indifferent homografts plotted against the time of application of the graft is indicated in Figure 2. Skin grafts placed immediately following marrow infusion seemed to survive somewhat longer although the numbers are too small to be significant. If skin was transplanted on day zero 9/20 died at eleven days if transplanted at days 21 71 8/10 died at ten days if transplanted at day 150 10/10 died at ten days (*)

It is interesting to observe the course of selected animals The only consistent pattern of skin graft rejection occurred in three animals who survived without objective evidence of marrow survival They were the only ones who rejected five of five homografts One of these animals incidentally rejected an indifferent homograft placed

( ) These ten animals have been studied by Dr Marcus Brooke & Dealy Jr for immunological function and will be reported elsewhere animals had 2-6 % female white cells after 600 r and 400 r and donor The ability to form circulating antibody against the sheep present in 8 9 The ability to develop delayed hypersensitivity present in 8 10 One animal lacked both circulating and delayed tion nevertheless it rejected the homograft promptly at ten days

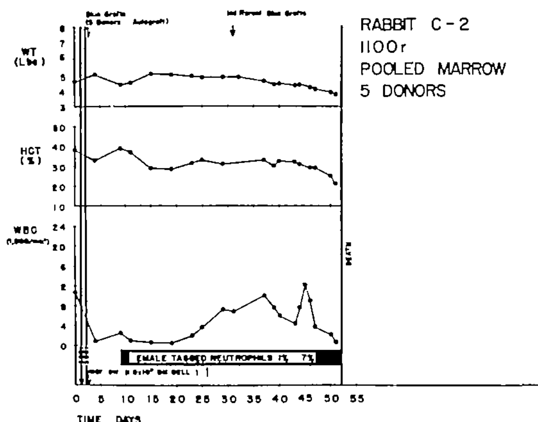


FIG. 3. — Animal C-2 Successful marrow transplant indicated by 1 %-7 % female tagged neutrophils from day eight to time of death at day fifty-one.

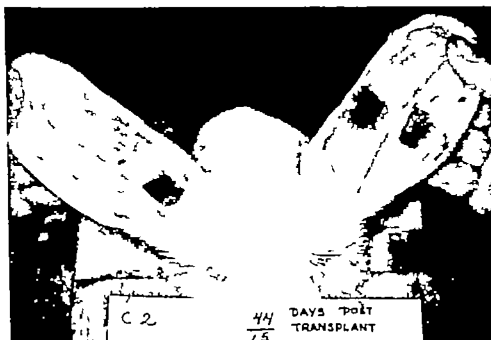


FIG. 4. — Same animal All seven grafts surviving at forty four days Three homografts on left ear (two white hair one black) and two homografts on right ear (one black and one white) Also on right ear are autograft (white near tip) and an indifferent graft fifteen days old (black at extreme right)

treated animals eight of fifteen indifferent grafts died by day eleven. Three were alive when the animals died at 15 22 days and one was still alive at day thirty-nine. Three indifferent grafts however were rejected late at 52 57 and 78 days.

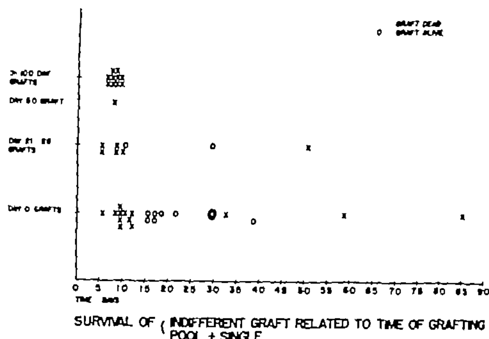


FIG. 2. — Survival of indifferent skin grafts plotted against day of grafting. Note that grafts on day zero tend to survive somewhat longer than those applied later but 73 % died by day ten. Total numbers are not significant.

The survival of indifferent homografts plotted against the time of application of the graft is indicated in Figure 2 Skin grafts placed *immediately following marrow infusion seemed to survive somewhat* longer although the numbers are too small to be significant. If skin was transplanted on day zero 9/20 died at eleven days if transplanted at days 21 71 8/10 died at ten days if transplanted at day 150 10/10 died at ten days (*)

It is interesting to observe the course of selected animals The only consistent pattern of skin graft rejection occurred in three animals who survived without objective evidence of marrow survival They were *the only ones who rejected five of five homografts.* One of these animals incidentally rejected an indifferent homograft placed at day -6

(*) These ten animals have been studied by Dr Marcus Brooke and Dr James B. Dealy Jr for immunological function and will be reported elsewhere. In brief all animals had 3-6 % female white cells after 600 r and 500 r and a single marrow donor The ability to form circulating antibody against the sheep red cell was present in 8/9 The ability to develop delayed hypersensitivity to tuberculin was present in 5 10 One animal lacked both circulating and delayed antibody production nevertheless it rejected the homograft promptly at ten days.

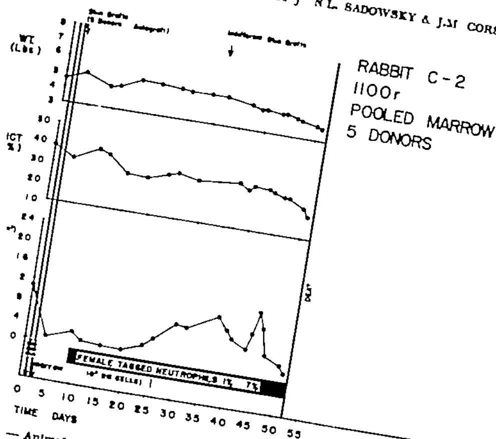


FIG. 3. — Animal C-2 Successful marrow transplant indicated by 1 %-7 % female tagged neutrophils from day eight to time of death at day fifty-one.

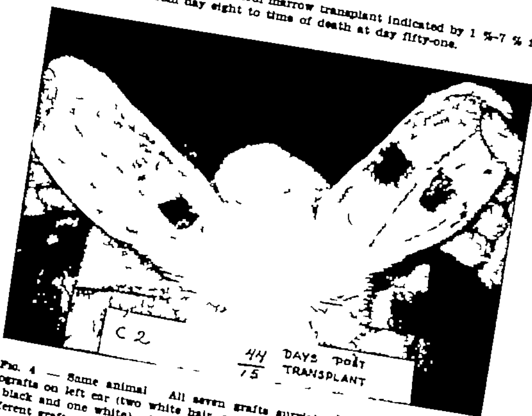


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at six days An animal with 4 6 % circulating female leucocytes with three intact homografts rejected indifferent skin homografts placed at day 29 and again at day 49 in ten and seven days respectively Another animal with circulating female leucocytes had five surviving homografts at its death on day 51 This animal had a surviving indifferent graft which had been placed at day 30 (Fig 3 and Fig 4).

A single marrow donor animal surviving beyond 100 days with intact homograft and autograft rejected an indifferent graft placed at day zero on day 78 A second indifferent graft (from a different donor) placed at day 20 lasted 52 days Thus both indifferent grafts were rejected within six days after a prolonged survival of seventy-eight and fifty-two days respectively

Discussion.

Rabbits were selected as the experimental animal in this study for several reasons. The dose of irradiation required for successful marrow transplantation is well established[3] the female chromatin label on the white cells is a convenient indicator of marrow survival[3] large areas of body surface are available for multiple skin grafts, and finally rabbits in a genetic sense are similar to man in their response to skin homografts.

The numbers of animals in our control series was not increased because of their general agreement with the findings of other workers. The test skin grafts on the first control series are similar to those of Medawar[4] The somewhat shorter skin survival time in our animals is a result of the selection of edema and swelling as the end-point Porter's study on non marrow treated irradiated rabbits with or without antibiotics is similar to the results of our second and third control series[7]

The plan for the experiments was suggested by the work of Hašek and Hašková[8] who were able to produce in newly hatched ducks an acquired tolerance by the use of an antigen collected from a pool of 20 60 neonatal and adult donors. The tolerant animals would subsequently accept skin grafts from some donors not included in the original antigen pool Varying the pool size altered percentage survival from indifferent donors. If 20 animals supplied the antigen 10 [illegible] of homografts from indifferent donors survived over eighty days if cells from sixty donors were in the pool 6/10 grafts from indifferent donors survived In animals given marrow from only one donor all homografts from other donors were destroyed by thirty days If ducklings with acquired tolerance after pooled antigen could accept skin grafts from non pool (or indifferent) donors we thought it conceivable that

lethally irradiated recipients sustained by an infusion of bone marrow from multiple skin donors might accept indifferent homografts Why animals made tolerant to pooled antigens accept an indifferent skin graft is not clear However the authors suggestion that « individual specificity is the result of the combination of a limited number of transplantation antigens » seems worthy of consideration. However in the lethally irradiated animals sustained by pooled bone marrow another explanation is possible. If the indifferent skin graft is applied before the infused pooled marrow begins proliferating in the new host the primitive stem cells of the new marrow multiplying in the presence of a foreign antigen might develop a type of tolerance, making it unable either to distinguish or to reject such a graft subsequently

Ashley et al [1] have used pooled antigens to produce an acquired tolerance which allows subsequently successful homotransplantation from donors separate from and not related to the original antigen donor group Using a leucocyte antigen in rats they describe 50 % of grafts applied at day 20 survived eighty-one days 31 % of grafts applied on thirty-day-old animals lived eighty-one days but no grafts applied at forty days survived. Their controls ranged about 8 % survival at 189 days for grafts applied at day 25 These observations may be interpreted as demonstrating that an acquired tolerance produced by non proliferating and therefore a transient antigen in this instance the leucocyte is not permanent.

Cannon Terasaki, and Longmire [2] have reported the production of an acquired tolerance to pooled blood antigens in chicks. These animals subsequently have increased percentage of survival of skin homografts from donors not included in the antigen pool. Although the tolerance produced is better (a higher percentage of skin grafts survived) when antigen and skin come from the same donor this requirement was not absolute Chicks which were test grafted at fifteen days with « indifferent » skin had 8 % viable homografts at twenty five weeks compared to 0 % of untreated controls Chicks grafted at two days had 16 % graft survival at twenty five weeks compared to 8 % for the untreated controls. The authors suggest some degree of adaptation of the homograft to the host under the conditions of their experiment

One important finding in our study is that more than one skin homograft can survive in an irradiated adult infused with pooled adult marrow Seventeen of twenty-one animals living beyond fourteen days had two or more viable homografts The assumption that skin homograft survival indicates marrow survival of that particular skin donor is supported by the observation that all homografts from the single marrow donors survived up to death of the animal some animals living

up to 100 days. If this is a valid assumption the use of pooled marrow does offer the method of increasing total marrow dosage. Dosage increase is not a problem in the experimental animals where pure strains are available or the donor may be sacrificed but it is a real hurdle in man. Unless one resorts to preservation of marrow with its uncertain and known limitations the only way to secure large amounts of suitable marrow is by the use of multiple donors. Extrapolation on a weight for weight basis of animal marrow dosage to man leads to this conclusion.

Further evidence that skin homograft survival indicates marrow survival comes from observations on the time of failure of homografts when failure does occur. The rejection time of 23.0 days in marrow treated animals without marrow survival corresponds to the rejection time in non-marrow treated irradiated controls. In contrast in animals with objective evidence of marrow survival and some successful skin grafts, the mean rejection time of these grafts which failed was 11.2 days. The regenerating marrow seems to be the agent causing the earlier skin rejection. The delayed rejection in the animals without marrow survival indicates that a high dose of irradiation by itself does not circumvent the homograft rejection phenomenon.

One wisp of clinical help may be derived from the meager data obtained from a study of indifferent skin homografts. The indifferent grafts placed at day zero seem to survive in greater number and for a longer time than those placed either at three to four weeks or at twenty five weeks as seen from Figure 2. However it must be noted that 73 % of all indifferent grafts died by the tenth day suggesting again that skin homograft survival requires specific donor marrow survival. Failure of indifferent graft survival might be related to the pool size our pool being only five whereas Hašek's and Ashley's groups ranged from ten to sixty.

SUMMARY

The use of pooled marrow and single marrow in the treatment of rabbits receiving lethally total body irradiation followed by skin homografts from each of the marrow donors has been studied. Some animals received an indifferent homograft from non marrow donor at varying times following marrow infusion. Nineteen of the forty animals (47 %) died by fourteen days. Sixteen of the remaining twenty-one had successful marrow grafts as indicated by the female chromatin marker on the circulating leucocytes. Nine animals (22 %) survived beyond twenty-eight days. All animals surviving beyond fourteen days with female leucocytes had 36 skin grafts surviving many having four or five. Animals surviving beyond fifteen days without evidence of marrow survival rejected all homografts. All ten animals receiving marrow from a single donor maintained their homografts from that donor to the time of death of the animal.

The number of indifferent skin grafts is too small to be significant although the survival pattern is interesting 22/30 or 73 % of homografts from indifferent donors failed to survive, although the pattern of surviving grafts suggests that indifferent grafts placed immediately after marrow infusion survive longer than those placed at three to four weeks or at twenty five weeks.

The survival of multiple skin grafts following use of marrow from multiple donors on irradiated recipients indicates survival of at least two and possibly four or five of the marrow sources. On the basis of these results in clinical practice it would seem more feasible to use multiple live donors of marrow one of whom could be a subsequent skin or kidney donor rather than attempt to produce tolerance to an indifferent antigenic source.

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DISCUSSION

LOUTIT Like Murray (and like Medawar yesterday afternoon) we have considered that lymphoid cells developing from stem cells could become tolerant. We have therefore given CBA mice 950 rads of X rays and restored them with CBA bone marrow At the same time or shortly thereafter we graft them with a skin homograft. If this is not too distantly removed within the *H* 2 system it can persist and function for a very long time—up to a year so far with, for instance, *C3H* as the donor of the homograft A-line skin has also on occasion lasted for many months but C57BL skin is shed more rapidly It is notable that isologous CBA/CBA chimaeras produced with marrow plus spleen are not so tolerant

MURRAY This observation of Dr Loutit's exemplifies the debt which we who are working with a pure homologous system as in man and the rabbit, owe to those working with pure strains of animals. The occasional prolonged survival of « indifferent » skin placed soon after marrow infusion in our series is probably explained by the development of a temporary tolerance by the proliferating stem cell The prompt rejection of the control skin homograft from the marrow donor fits in with this explanation

BERRIAN The following observation may be of interest in regard to the survival difference between the successfully and unsuccessfully transfused marrow hosts *Using A mice as donors and untreated C57BL as* recipients, Dr McKhon and I have observed (*Ann Surg.*, in press) that injection of spleen cells intraperitoneally on the day of or within 48-7 hours after skin grafting immunises the hosts to these primary skin grafts the strength of the immunity decreasing with prolongation of the interval.

MURRAY Thank you for your interesting observation Why a dose of X ray sufficient to allow a successful marrow graft will not allow a skin graft is probably related to the route of antigen administration and its rate of contact with the host RE system. Intravenous marrow can establish its blood supply more rapidly than skin which requires at least 72 hours.

OWEN You spoke of the immunological status of the treated rabbits indicating for example that anti-sheep red blood cell antibody was *produced by them. Were the titres observed comparable to those in* normal rabbits or was there a quantitative reduction in titre comparable to that found by Makinodan et al. in mice retaining homologous or heterologous marrow transplants

MURRAY The titres followed no definite pattern but did reach normal range The highest titres were 20,480 and the lowest 640. Only one had none Other immunological parameters including leucocyte phagocytosis and tuberculin sensitivity could not be correlated all animals having varying degrees of response All however rejected indifferent skin placed at 150 days.

GORER One very minor point We inoculated irradiated pure strain *mice with a mixture of two pure* strain bone marrows We had some losses which were probably infective however our cytotoxic tests showed that the spleens contained cells from only one f the donor

MURRAY The results described by Dr Gorer were more or less anticipated by us in our study However it does seem that in our test system, several sources of marrow can exist comfortably for a while.

HAŠEK I congratulate Dr Murray on his development of a clinical model of transplantation tolerance in which the strategy of the experiment is directed towards the solution of a clinical problem In our experiments on the injection of pooled immature duckling cells into newly hatched ducklings—animals with a long adaptive period—we found tolerance both to the donor skin and to skin from ducks which did not belong to the donor pool. Perhaps the rabbit, in which it is difficult to produce tolerance, has either too short an adaptive period or the donor pool is as you yourself suggested not large enough

BRENT In theory it would seem desirable to inject marrow cells from a *single* donor I am thinking particularly of the observation mentioned by me in the discussion of Trentin's paper that the injection of a mixture of homologous cells (C57 and CBA in this instance) into newborn A mice will lead to a *graft-versus-graft reaction* resulting in the elimination of the cells of one strain (C57) In this particular experiment, Billingham and I could be certain of this interpretation because the recipients were fully protected against the normally lethal effect of the C57 cells and because the recipients—though fully tolerant of CBA skin grafts—proved to be weakly immune in respect of C57 grafts.

MURRAY Unfortunately there are no isologous strains of human beings. We were aware of the potential hazards of « graft versus-graft » reactions but adopted this plan purely in an effort to increase fresh marrow dosage without resorting to methods of preservation Of course a single marrow donor would be far preferable if sufficient marrow is procurable. Dr Mathé's report here today indicates that it might be.

LOUTIT This is really a comment on Brent's and Gorer's observations on graft versus-graft reactions. These results may not necessarily be immunological reactions. Physiological competence may enter into the problem. We have noted that CBA (CBA × T6) F_1 chimaeras can be reversed by giving CBA spleen cells which recolonise the chimaera not apparently by reacting immunologically against T6 cells but by being more competent physiologically in a number of animals a few T6 cells can be shown by cytological examination to persist and CBA lymph node cells did not effect the reversion to CBA in similar chimaeras.

MURRAY To illustrate the decisions clinicians are forced to make *without having experimental data available I would like to amplify* remarks made earlier regarding our « non identical » twin patient who has recently had a kidney transplant. The close immunologic and genetic compatibility of the twins (demonstrated by skin graft survival on the sick twin, while the healthy twin responded with a second-set response to a reciprocal homograft) may resemble that of Odell's C and D strain rats which had successful marrow transplants following sublethal X-ray dosage. Therefore we selected a similar dose range of radiation. Another decision remained regarding the timing of the kidney transplant. For this we relied on observations made by Dr M. Brooke on our rabbits which were skin grafted at varying periods following sublethal X ray treatment. Grafting 24 hours after X-ray treatment seemed optimal with wide individual variation at all times. His observations are not completed nevertheless, clinical circumstances forced our hand.

May I add a final comment ? We must not assume that secondary disease will occur in man following marrow therapy Certain mice strain combinations do not get it in rabbits only about 30 % develop *the syndrome. At least 50 % of rabbits live quite well following total* body irradiation and marrow infusion not in perfect health but at least with some immunological capacity We would be happy to accept such survivors in the treatment of some discouraging diseases, like cancer of the stomach or lung if only such therapy were applicable (which of course it is not).

Does a total transfusion done shortly after birth induce a state of tolerance in receptor ?

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The experiments done by Medawar [1] and his school have shown that if baby mice are injected with homologous spleen cells before or very shortly after birth they tolerate a skin homograft from the same donor or from a mouse of the same strain

The percentage of tolerant mice decreases rapidly if the injection is not done within the first few hours after birth [6]

Woodruff [7] on the other hand drew the attention of research workers to the fact that, in the rat, tolerance may be induced even several days after birth The period of immunologic immaturity required to create this state of tolerance seems to vary very much from one species to the other and on this occasion Woodruff [8] wondered whether it was possible to induce this tolerance in a child after birth In two instances, after an intramuscular injection of leucocytes, he had obtained partial tolerance to a skin homograft from the same donor

Most experiments on animals had been done with living spleen cells, the latter being expected to live and multiply in the organism of the host.

But at this point a serious question arose if the donor and receptor mice were too different genetically there was a risk of seeing the injected mice die, after having undergone a deep change in their general health (Runt disease) (Billingham & Brent [3]) These deaths were

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attributed by various authors, and particularly by Simonsen[4] to an immunological reaction of the injected homologous cells which develop antibodies against antigens of the receptor

To avoid serious even fatal accidents, due to what has been called « graft versus host reaction » Simonsen suggested using for previous injection, embryonic cells still immunologically immature.

It seemed as though this would be very difficult to apply to man

And yet for a few years now one has been doing more and more often and one might even say more and more easily total transfusions to new-born babies, in the hope of saving them from erythroblastosis (by foeto-maternal incompatibility)

It is known on the other hand that intravenous injections of blood may confer to the animal a certain tolerance to homografts less spectacular however than that induced by spleen cells. It must be borne in mind that in experiments on mice, the number of cells injected in the form of blood is relatively small Medawar[2] has insisted on this matter of concentration

In total transfusions carried out for congenital erythroblastosis, the amount of blood injected to the new born baby is enormous, as the object is to replace as completely as possible his own blood by that of the donor The number of homologous white cells thus transfused reaches milliards.

If this transfusion is done early enough it seems one might have in hand the favourable conditions required to create a tolerance to homografts.

But before approaching this problem it is interesting to note the relative innocuity of this enormous transfusion of homologous cells No « graft versus host reaction » seems to occur the children thus treated present no « runt » and develop normally except for eventual lesions due to their erythroblastosis.

Of course for these total transfusions the donors are selected in such a way that there is a maximum similarity between their blood groups and those of the receptor—which does not prevent Rh+ children being injected with Rh— blood.

What becomes of the homologous cells? Do they keep on living and multiplying in the organism of the receptor If so for how long

These questions cannot be answered yet

It was however interesting to verify the influence of a total transfusion on the fate of a homograft from the same donor The interest was all the greater as at the beginning of 1958 Puza & Combos had published the result of their experiments on dogs

With total transfusions of a quantity of blood equal to ± 2 × 7.6 % of the total weight of the animal done towards the third or fourth day after birth sometimes followed by a second transfusion towards the twelfth day these authors have obtained a tolerance of varying quality and duration sometimes even with apparition of hairs on the homografts.

On one hand we therefore tried to reproduce the above mentioned experiments on the dog and on the other hand to study the evolution of a homograft in children who had been totally transfused.

I — Experiments on the dog.

When puppies were born in our laboratory one of us (*) reproduced these experiments two or three days after birth, with a view to performing a total transfusion at a rate comparable to that used in human clinic. The object was to appreciate afterwards tolerance or intolerance to skin homografts from the blood donor.

To obtain as closely as possible the same conditions as those in which children with erythroblastosis were treated, the first precaution was to choose the donor in such a way as to eliminate the factor of blood incompatibility. In our experiment, the donor was a large dog with short black fur the receptor a crossbred dog with very light coffee coloured fur. The blood from the donor was taken at the femoral artery —with all aseptic precautions—and collected into a sterile flask containing 10 cc. physiologic saline heparinized at 10,000 I.U.

The total transfusion was done without too much difficulty by introducing a small polythene tube into the central end of the femoral vein of the receptor. A needle, fixed to a polythene tube was introduced into the central end of the femoral artery.

The femoral artery pours its blood into a graduated glass. The blood of the donor is injected into the femoral vein after a previous injection of 5 cc of physiologic saline containing 5,000 I.U. heparin. 52 cc of blood are withdrawn from the receptor and he is injected with 50 cc from the donor.

The mother refused to care for her puppies after we had handled them during the second series of experiments and we were able to keep only one small dog alive. Here is the complete observation of the latter.

Experiment on a totally transfused dog.

The dog grows normally and on the 54th day he weighs 4.500 kg. Under general anaesthetic (25 mg Themalon given subcutaneously) we

(*) G. Lejeune-Ledant.

placed on the left lateral side of the thorax skin grafts from the blood donor (one auto- and two homografts) on beds prepared in advance. They were fixed to the skin of the receptor with some oooo silk stitches. The grafts were placed in such a way that the hairs were inversed when compared to those of the receptor Dressing with tulle gras, one dry compress and plaster cast

On the 8th day the dog caught at the dressing and scratched the grafts. It was difficult to verify the exact state of the grafts.

On the 12th day there was a certain degree of infection but the greater part of the grafts was in a good state

On the 15th day a few black hairs appeared at the same time as a little hyperkeratosis.

On the 19th day some black hairs still persisted

At this time, and because of the incident which occurred on the eighth day and might have unfavourably influenced evolution we decided to do the same operation on the right side of the thorax (one auto- and two homografts)

Same dressings but in this instance with reinforced plaster cast In fact here, it was a second graft which in a normal dog should cause a « second set reaction » For this reason we first looked at the grafts on the sixth day and found the three of them perfectly living and supple.

On the 12th day the three grafts were in the same state and we removed the stitches. Both homografts subsequently behaved like the autografts they were more pigmented but at no time did hairs appear

After four months, the situation is unchanged

Conclusion

In a young dog totally transfused at birth skin homografts from the blood donor behave practically as autografts, with the sole difference that, as opposed to autografts homografts do not grow hairs or if they do the hairs are very scarce and transitory

More so not only do a second series of homografts give no « second set reaction » but they show exactly the same characteristics of vitality and retain their pigmentation without however growing hair all over Four months later the situation remains unchanged.

May we conclude that there is a total tolerance We cannot be sure and further experiments will perhaps enable us to be more precise on the subject.

In any case our experiments quite confirmed the results obtained by Puza & Gombos and we considered ourselves authorised to carry out experiments on former erythroblastic patients totally transfused at

birth. The trial was all the more justified as in the event of favourable results, the consequences could be particularly worthwhile for these children.

II — Skin homografts in a child 6 ¾ years old transfused at birth.

With respect to children transfused at birth we would have liked to graft a greater number of them with skin from their former donors. Although it would be relatively easy to find donors who would accept this small operation it would be less easy to get voluntary receptors

Most children, totally transfused at birth in our countries are at the most six or seven years old At the time of, and subsequent to their erythroblastosis, these children gave serious concern, and most parents hesitate to impose on them the slightest operation which is not absolutely necessary Up to now we have only had one case. We did not choose the child and if we had had several children at our disposal we would not have chosen that particular one. But we were fortunate enough to deal with particularly understanding parents and with a very reasonable child. Parents and child understood the eventual interest of what we proposed to them, and they deserve, as well as the voluntary donor all our admiration and gratitude.

Case report.

The baby boy was born on October 21st 1951 at eight months, with erythroblastosis. He was transfused with Rh— blood at the 40th hour after birth. At that time, of course, the umbilical vein was badly suited for the transfusion which proved difficult. 160 cc blood were withdrawn and 210 were injected

The child received a second transfusion of a second donor of 80 cc on October 31st, and a further one of 90 cc, of a third donor on November 16th.

From an absolute viewpoint, the case was not treated under ideal conditions. The transfusion was done at the 40th hour only and in view of the quantities of blood withdrawn and injected one cannot talk of a real total transfusion. In fact, 160 cc blood from the child have been replaced by 210 cc of blood from the first donor Successive fractionated red cell counts revealed approximately 2/3 Rh— red cells and 1/3 of Rh+ red cells

Operation on June 4th 1958

Donor Very willingly allowed a skin graft to be taken under local anaesthetic A fragment of total skin 2 cm × 1 cm was taken from

total and to study conditions and reasons of eventual degrees in acquired tolerance.

Post scriptum All the question considered in our paper was based on the induced tolerance of a strict individual specificity as it is the case for all the artificially acquired tolerances Both injected cells and homograft must come from the same donor Now a new fact has at this instant arisen in the case of the child previously mentioned. The paper being ready for printing Prof. Moureau nevertheless decided to reexamine once again all the elements of the problem. The date of that total transfusion corresponded, in fact to a transitional period during which he became Chief Director of the « Centre de Transfusion de Liège » At this time, his new system of classification was not yet in operation. This re-examination disclosed the following results

For the total transfusion in question two donors had been selected, a man and a woman Each of these supplied 500 ml of their blood In fact only one of the two blood containers was used. Since the man remained all the night close to the family of the child everyone felt certain that the blood used was his. The results of an extended and detailed search has proved, beyond any doubt that the blood given was that of the woman. Since then this woman died We are quite certain now that the donor of the skin homograft was not the previous blood donor Because of this unforeseen event we were about to renounce giving the entire paper until a more extensive experiment could be undertaken.

Afterwards, we have, on the other hand thought that this inversion of donors had put us under unexpected experimental conditions and that it should be an error not to consider this peculiar case with more interest Discoveries sometimes of extreme importance have been the consequences of fortuitous events. One must have the courage to mention these unexpected circumstances.

If we consider in the light of this new fact the details of our experiments on the child we notice a certain number of points which merit our attention

In 1951 the child born with congenital erythroblastosis, was submitted in his 40th hour of life to an attempt of total transfusion The operation extremely difficult in this particular case, was limited to an injection of 210 ml of blood of a woman 160 ml of blood of the child having been removed.

But since the baby was and remained very anaemic, two additional transfusions were performed on the 10th and 26th days of his life Each time the blood was supplied by a different donor

This baby had thus received after removal of part of his own blood three successive blood transfusions from *three different* donors.

Six and a half years after birth the parents of this child agreed that an attempt of skin homograft be performed. Always guided by the concept of individual specificity of all induced tolerance we chose the presumed donor of the first blood transfusion.

Actually the blood of this man had never been used for any of the three blood transfusions. The transplanted skin homograft was that of a stranger who has nothing in common with the receptor other than belonging to the same blood group.

In spite of that we observe, if not a complete or total tolerance at least an extremely long survival perhaps permanent for one part of the graft.

We could not stop thinking of the experiments of Cannon, Terasaki and Longmire[4] who realised a partial tolerance by injection of embryo pooled blood in certain chicken embryos of different breeds which have « mutatis mutandis » a certain analogy with our observation.

Of course, it is impossible to draw conclusions from one single case. But the facts observed in this case merit our attention calling for more control.

Different factors may play their role in a prolonged survival like that we observed in our case. Agammaglobulinemia is out of question: this boy had never an infection; electrophoresis of his serum is normal and so the rate of his urea.

Our boy could be one of these exceptional cases of very rare individuals who tolerate for an uncommon long time a skin homograft of an unrelated donor. This could be verified by the fate of another skin homograft from a donor chosen at random. But that new assay would not be easily accepted neither by the boy nor his parents.

What about the relation between the blood groups of the donors and recipient? In his communication titled *Genetics of homotransplantation in human,* Rogers concluded: « When gross compatibility of all the major and minor blood groups and subgroups known to day exist between a donor and a recipient skin Hg transplanted under control conditions between this donor and recipient survive longer than skin Hg obtained from donors with dissimilar or incompatible blood groups and subgroups ».

Whatever the influence of these similarities of blood groups may be, the tolerance of the homograft in the case of our boy seems to be too important to find its explanation in that similarity only.

Different blood groups and subgroups of the boy, the three blood donors and the graft donor

Receptor

J M O anti A anti B, MN $\frac{DCe}{dce}$ p Kell neg Fy^{a}neg Le^{a}pos.

Blood donors

1 La O anti A anti B N $\frac{dce}{dce}$ p Kell neg., Fy^{a}neg., Le^{a}neg

2. Mu O anti A anti B, M $\frac{dce}{dce}$ p Kell neg., Fy^{a}neg., Le^{a}neg

3. Or O anti A anti B $\frac{dce}{cde}$
(deceased donor).

Graft donor

Z O anti A anti B, MN $\frac{dce}{dce}$ p Kell neg., Fy^{a}neg Le^{a}pos.

The injection of a very great number of white cells in the early days after birth may also have played a role, perhaps more essential in this tolerance.

To answer all these questions, many controls will be necessary But the problem is important. If really a child polytransfused at birth with blood of a great number of donors, could become tolerant at least partially to skin homograft Hg of any individual belonging to the same blood groups as his multiple donors, there would be a real interest to do these early transfusions with pooled fresh blood

It is rather difficult to advance rapidly in a problem where all depends of the understanding and goodwill of so many combinations of persons. Two other cases are now under observation. We hope to have in the near future some more results perhaps able to shed a little more light on this complicated but very interesting problem.

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DISCUSSION

WOODRUFF I should like to ask whether there had been any delay in Dr Albert s cases between collecting blood from the donor and transfusing the newborn recipient. In the one similar case we studied this interval was not known, and might conceivably have been long enough to account for our failure to produce tolerance. Having found that skin homografts exchanged between two men whose red cells were undistinguishable in respect of all the antigens known in 1951 were destroyed in the normal time, I personally feel that the long survival of the homograft in Dr Albert's case was probably due to the fact that the recipient was transfused soon after birth rather than to the similarity of red cell antigens of the donor and recipient.

ALBERT To answer Dr Woodruff's first question, we never use preserved blood for the total transfusion in the newborn erythroblastic children The blood donors are asked to come straight away—even before the birth of the baby if one can foresee the case—and 500 cc of blood from each of the two donors are collected immediately before transfusion. By proceeding in this way there is no delay between collecting blood and the transfusion of the baby and we may be sure that the injected cells are still in perfect condition. As to the second question our opinion is that the similarity of blood groups between adults is not sufficient to produce in all cases a prolonged survival of the homografts.

HAŠEK In the experiments of Puza and Gombos on dogs a high degree of tolerance was obtained in some but not all cases. In order to obtain a maximum number of viable leucocytes in the transfused blood the donors are bled several times at certain intervals before the time of collecting the blood for transfusion This might have favoured the induction of a higher degree of tolerance in their experiments

ALBERT Thank you Dr Hašek this is no doubt a factor that must be taken into account in this kind of work

The use of histocompatibility genes as markers for the study of isoantigenic variation in populations of tumor cells

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Proposed System

It has been suggested[17] that histocompatibility genes may be useful as markers for the study of genetically determined intercellular variation in populations of tumor cells. In particular heterozygous F_1 hybrid hosts produced by the crossing of two of the isogenic resistant (IR) mouse lines of Snell[31] have been recommended[18, 20] as tumor donors. The IR lines have been bred with the purpose to establish a coisogenic background while maintaining an allelic difference at one of the histocompatibility loci. Theoretically they are homozygous with regard to their entire genome, except the histocompatibility gene in question and a chromosome section of undefined length around it. It is difficult to say how well this theoretical expectation is fulfilled in reality; the existing IR lines are probably good approximations only. Provided that the number of major histocompatibility genes differentiating the two lines used for the cross were not more than one, tumors induced in the F_1 hybrids would be particularly suitable to detect isoantigenic variation at the cellular level since they might be expected to give rise to variant cells, selectively compatible with one or the other of the parental strains either by mutation or as a result of phenotypic changes involving the inactivation or loss of the isoantigenic components specifically derived from the opposite parental strain.

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We have been studying tumors induced in F_1 hybrids of this type during the last two years. Previous reports on this work[9 12 13 14] have included details of the transplantation tests and the serological and cytological studies. The purpose of the present paper is to discuss these and some other previously unpublished data, and to consider the problems that remain to be solved In particular the following questions will be dealt with

1 Are isoantigenic variants, compatible with a given host genotype, detectable even when intermixed with a very large population of genetically incompatible cells?

2. Is it possible to extract clear-cut isoantigenic variants from various tumors by utilizing the homograft reaction of coisogenic resistant hosts as selective force and to distinguish such variants from « false positives » growing in the presence of and in spite of a homograft reaction ?

3 What types of isoantigenic variants can be obtained and in what frequencies?

4 How permanent are the changed isoantigenic characteristics of a variant subline after it has been returned to its original host genotype?

5 Is there any evidence of genetic recombination during the growth of two different isoantigenically marked tumor lines in mixture?

6. What can be said about the nature of the cellular change underlying the formation of variants?

1 *Detectability in reconstruction experiments*

The detectability of a few compatible isoantigenic cell variants in large tumor-cell populations, incompatible with and exposed to the homograft reaction of a coisogenic resistant host, would be greatly hampered if the variants were damaged concurrently with the destruction of the majority in a non-specific way This eventuality was studied by model experiments[12] A small number of sarcoma cells derived from strain A mice were mixed with large numbers of cells derived from a similar sarcoma induced in an A × A.SW F_1 hybrid mouse, differing from the parental A type by the presence of the histocompatibility factor H-2 The F_1 hybrid tumor chosen was very specific and consistently refused to grow in mice of the parental A strain, even when inoculated in large numbers When compatible A sarcoma cells were randomly mixed with F_1 cells in frequencies between $4 \cdot 40 \times 10^{-7}$ and the mixture was inoculated into A mice, the compatible cells grew not only as well but better than when injected alone in the

same number. Thus the specificity of the homograft reaction was sufficiently high to distinguish between compatible and incompatible cells, in spite of the fact that compatibility was probably a matter of a single histocompatibility factor difference only and the compatible cells were intermixed with the incompatible ones in a random way. Its selectivity was powerful enough to concentrate the compatible cells from their very low initial frequency to a pure culture and some factor involved in the destruction of the incompatible cells actually stimulated the growth of the compatible ones. The latter finding has been a regular experience with several different host tumor systems [14,33] and seems to be analogous with the growth stimulating effect exerted by irreversibly X-ray inactivated tumor cells on the growth of admixed compatible cells, described by Revesz [34]. There was only one exceptional tumor type where inhibition was sometimes found instead of stimulation, namely in the case of certain lymphomas [24]. With the possible exception of this material, the model experiments thus justified the expectation that possible isoantigenic variants would be recoverable from populations of tumor cells that have originated in F_1 hybrid hosts produced by the crossing of two isogenic resistant lines.

2. *Isoantigenic variants and false positives*

Sixty nine methylcholanthrene-induced sarcomas, originating in various F_1 hybrid combinations of strain A mice and the three coisogenic resistant lines [35] A.SW ($H-2^a$ $H-2^s$), A.BY ($H-2^a$ $H-2^b$) and A.CA ($H-2^f$ $H-2^a$) were tested for their ability to grow in the parental strains. Various types of behavior could be observed. A few sarcomas were « non-specific » either immediately upon their origin or after a certain number of serial passages. They grew indiscriminately in both parental types and also in foreign genotypes. They grew less well or not at all in mice of the parental strains preimmunized against the isoantigens of the opposite parent, or in foreign genotypes preimmunized against the hybrid genotype of the tumor.

The great majority of the sarcomas tested were highly specific in their transplantation behavior and grew regularly in the F_1 hybrid genotype of origin but only seldom or not at all in any of the parental strains. Occasional takes in parental strain mice were of great potential interest for the present study and such tumors were regularly tested by further transplantation into both parental strains and into the other two coisogenic resistant lines as well together with various F_1 combinations between these and the two parents. Different types of behavior could be observed. Some tumors failed to grow upon repeated testing in the same parental genotype in which they appeared. Others grew in a cer

tain often high proportion of the same parental strain but not in the other parent or in foreign genotypes. Tests in preimmunized mice gave interesting results. Some tumors were fully compatible with their new parental host even if preimmunized by one or several inoculations of normal or malignant tissues from the opposite parental strain Others grew only in non immunized mice and regressed in preimmunized animals When lines of the latter type were carried in non immunized hosts of the parental strain during a series of transfers, one of two alter natives could happen the line died out after a number of passages failing to take even in non immunized hosts, or it became increasingly and sometimes fully compatible with preimmunized hosts. The latter development was observed repeatedly with certain host-tumor combin ations and will be discussed in more detail in the next chapter

Occasional takes were sometimes obtained in coisogenic resistant mice outside the two parental strains (e.g with A × A SW F_1 tumors, in A BY or A.CA mice or in various F_1 hybrids derived from crosses between A BY or A.CA and one of the parental strains) In spite of extensive testings, it was never possible to establish a subline that would « breed true » in these partially foreign genotypes Upon repeated test ing they either refused to grow at all or turned out to be « non-specific » and grew in a wide variety of strains similarly to the tumors that were non-specific from the beginning Even in this case, growth in foreign genotypes could be usually prevented by previous immuniz ation.

Some experiments were carried out in order to see whether a variant subline that was already selectively compatible with one of the parental strains could also be adapted to the opposite parent Whenever takes were obtained in the opposite parent, they turned out to be « non-specific » forms on further passage, capable of growth in foreign genotypes also Experiments are presently in progress in order to establish what (if any) H 2 isoantigens such « double selected » tumors contain. It may be pointed out that while it was frequently possible to select clear-cut variant sublines specifically compatible with one or the other of the parental strains, from the same F_1 tumor no line could be established so far that would have been compatible with both parental strains to the exclusion of foreign H-2 genotypes.

A series of similar tests were performed with eleven sarcomas induced in an identical fashion in homozygous mice of the strains A A.SW or A.CA Takes were much less frequently obtained with such tumors in coisogenic resistant mice carrying foreign H 2 alleles than the case was when heterozygous F_1 tumors were tested in one parental strain. Whenever such occasional takes were obtained they failed to

same number Thus the specificity of the homograft reaction was sufficiently high to distinguish between compatible and incompatible cells, in spite of the fact that compatibility was probably a matter of a single histocompatibility-factor difference only and the compatible cells were intermixed with the incompatible ones in a random way its selectivity was powerful enough to concentrate the compatible cells from their very low initial frequency to a pure culture and some factor involved in the destruction of the incompatible cells actually stimulated the growth of the compatible ones. The latter finding has been a regular experience with several different host tumor systems [11 12] and seems to be analogous with the growth stimulating effect exerted by irreversibly X ray inactivated tumor cells on the growth of admixed compatible cells, described by Revesz [23] There was only one exceptional tumor type where inhibition was sometimes found instead of stimulation namely in the case of certain lymphomas [12] With the possible exception of this material, the model experiments thus justified the expectation that possible isoantigenic variants would be recoverable from populations of tumor cells that have originated in F_1 hybrid hosts produced by the crossing of two isogenic resistant lines

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Occasional takes were sometimes obtained in coisogenic resistant mice outside the two parental strains (e.g. with A × A.SW F_1 tumors in A.BY or A.CA mice or in various F_1 hybrids derived from crosses between A.BY or A.CA and one of the parental strains). In spite of extensive testings it was never possible to establish a subline that would « breed true » in these partially foreign genotypes. Upon repeated testing they either refused to grow at all or turned out to be « non-specific » and grew in a wide variety of strains, similarly to the tumors that were non-specific from the beginning. Even in this case, growth in foreign genotypes could be usually prevented by previous immunization.

Some experiments were carried out in order to see whether a variant subline that was already selectively compatible with one of the parental strains could also be adapted to the opposite parent. Whenever takes were obtained in the opposite parent, they turned out to be « non-specific » forms on further passage capable of growth in foreign genotypes also. Experiments are presently in progress in order to establish what (if any) H-2 isoantigens such « double-selected » tumors contain. It may be pointed out that while it was frequently possible to select clear-cut variant sublines specifically compatible with one or the other of the parental strains, from the same F_1 tumor no line could be established so far that would have been compatible with both parental strains to the exclusion of foreign H-2 genotypes.

A series of similar tests were performed with eleven sarcomas induced in an identical fashion in homozygous mice of the strains A, A.SW or A.CA. Takes were much less frequently obtained with such tumors in coisogenic resistant mice carrying foreign H-2 alleles than the case was when heterozygous F_1 tumors were tested in one parental strain. Whenever such occasional takes were obtained they failed to

« breed true » in the new genotype and either died out after one or a few passages, or showed a highly non-specific behavior

This experience with homozygous and heterozygous tumors indicates that we are dealing with at least two different phenomena. One *probably corresponds to what Snell describes as « false positives » where the tumor grows in the presence of and in spite of a homograft reaction and where its growth can be at least partially prevented by testing it in preimmunized hosts*[24, 27]. This result is not particularly frequent on the whole and the liability of its occurrence varies from tumor to tumor. It may occur with both homozygous and heterozygous tumors and also with the selected and originally specific sublines of the latter. It does not seem to be limited to the parental *strains or to certain specific genotypes* although it may be obtained with greater *ease in one genotype than in another. The latter variation may be* attributed to variations in the strength of the homograft reaction depending on the nature of the isoantigenic barrier between tumor and host.

The appearance of variants selectively compatible with one of the parental strains appears to be a different phenomenon for the following reasons

A certain percentage of takes was obtained in one of the parental strains. When tested further some of these tumors were selectively compatible with the parental strain in question but not with the opposite parent or any other strain. They grew in preimmunized mice of their new strain to a varying extent but in a number of cases they were compatible even with such hosts, either immediately or after one or a few passages in non immunized mice. From certain tumors it was possible to establish both kinds of variants selectively compatible with the maternal or the paternal strain resp., but still refusing to grow in the opposite parent. This result could be only obtained with heterozygous tumors and was restricted to the parental strains excluding *other coisogenic resistant mice carrying* foreign alleles at the H-2 locus, and other F_1 hybrids differing from the genotype of origin with regard to one *substitution at the H-2 locus.*

The question arose whether such selectively compatible variants grow in their parental hosts because they have lost the specific isoantigenicity determined by the H-2 factor derived from the opposite parental strain. This was tested in a number of different ways. The original tumor and its variant(s) were compared for their ability to induce the formation of hemagglutinins[4] and cytotoxic antibodies directed specifically *against the H-2-determined* isoantigenic products of both parental strains[2, 13, 15] for their ability to absorb performed hemagglutinins

or cytotoxic antibodies from isoimmune sera and for their ability to provoke a « second-set response » subsequent to the inoculation of heavily irradiated cells into the variant-compatible type tested by challenging the pretreated mice with another F_1 hybrid tumor derived from the same genotype and capable of temporary growth in the parental type in question. In addition our co-worker K. E. Hellström has studied the sensitivity of F_1 hybrid lymphomas and their variants towards various isoimmune sera by the direct technique of Gorer and O'Gorman [8].

All techniques gave essentially similar results. Variants which were able to grow in a high frequency of hyperimmunized mice of their new parental genotype did not seem to contain detectable amounts of isoantigens derived specifically from the opposite parental strain. Less clear-cut results were obtained with variants that grew in non-immunized but not in preimmunized mice but the opposite parental antigens were still detectable in several cases by the sensitive method of provoking a second-set response with preirradiated cells and by the direct cytotoxic test on the lymphoma cells. In two cases subsequent serial transplantation in non-immunized mice of the parental genotype led to an increased ability of such variant cells to grow in preimmunized mice. When tested again at this stage, the specific antigens of the opposite parental strain were no longer detectable with either technique.

In summary specific variants can be distinguished from « false positives » by their selective compatibility with one of the parental strains including the ability to grow in hosts hyperimmunized against the opposite parental type, and the apparent loss of H-2-determined isoantigens specifically derived from the opposite parent. Such variants have been only found with F_1 tumors so far and were limited to one of the parental strains to the exclusion of other coisogenic resistant mice, carrying a foreign H-2 factor. « False positives » are less discriminative in their host requirements grow in many different genotypes to a varying extent do not breed true to type on selective transfer and are completely or partially inhibited upon testing in preimmunized mice. They occur with homozygous as well as with heterozygous tumors and are not limited to the parental strains. The tendency to give rise to false positives varies considerably from tumor to tumor [2, 12, 24, 27].

3. *Types of variants obtained*

For each F_1 hybrid tested one of the parental strains turned out to be a more frequent site of variant formation than the other. At the time of our previous report [14] it appeared that the maternal strain

was usually preferred Testings on reciprocal hybrids were not available, however and in three of the four *crosses studied until* that time, the paternal genotype was the same, namely the original A/Sn strain from which the three *other cosogenic* resistant lines had been derived [21, 23] Several alternative explanations were considered [13] one of which was the possible existence of self reproducing particles with isoantigenic activity derived from the mother This possibility was excluded by more recent experiments, however which showed that the « favored » strain of variant formation remained the same for a given F_1 genotype even if tumors induced in the reciprocal hybrids were tested. Several alternative possibilities remain to be considered, but the difference observed is most probably *due to the fact that the isoantigenic* barriers separating the tumor from the two parental strains differ in number and/or strength making the development of compatibility with one parental strain less likely than with the other If the barriers are different in number a greater number of changes have to occur in one direction than in the other The finding that variants develop by at least two steps in a number of cases enabling growth first in untreated and only later in preimmunized mice can be interpreted to mean that several barriers have to be transgressed It has *been shown* [1, 21] *that weak histocompatibility differences are often not* expressed unless preimmunized hosts are being used If the barriers *are different in strength the period of « background growth » of* the original incompatible tumor previous to its regression may be of different length The fact that *one passage through newborn mice* may facilitate the extraction of variants growing in non immunized mice, and that passage through non-immunized mice enables the establishment of variants compatible with preimmunized mice may be interpreted as indicating the importance of the length of the « background growth period » Experiments are now in progress to distinguish between these possibilities. Meanwhile it can be pointed out that in all three crosses where strain A entered (A x A.SW A x A.BY and A x A.CA) this was always the *less* frequented parental type with respect to variant formation It may be recalled that the three isogenic resistant strains A.SW A.BY and A.CA have all been derived from crosses of strain A with another unrelated strain This was followed by repeated backcrossing to A and selective elimination of the segregants containing H 2 (derived from strain A) by *challenging them with a strain A tumor* The negative survivors were always bred further in order to retain the H 2 factor derived from the other foreign strain simultaneously with the introduction of more and more of *strain A* genetic background It be excluded this proce

dure may fix more than one histocompatibility factor derived from the foreign strain used for the original outcross.

Considering now the formation of specific variants from a given tumor-cell population it may be said that different types of variants can often be obtained from the same tumor. One example of this has been already mentioned: some tumors give rise to a certain proportion of variants in *both* parental strains, each of which breeds true upon further subpassage and remains selectively compatible with the parent where it has been selected to the exclusion of the opposite one. By the use of suitably selective hosts, the system can be further refined and additional types of variants can be selected. To mention one example, the MSWB sarcoma induced in an A × A.SW F_1 hybrid host has been found to give rise to variants compatible with the A.SW parental strain repeatedly [11] and it could be shown that many of these variants arise independently from each other [8]. It is known [9] that the A strain ($H\text{-}2^a H\text{-}2^a$) differs from the IR line A.SW ($H\text{-}2^s H\text{-}2^s$) with regard to 2 isoantigenic components at least. Strain A mice can be characterized by the H-2-determined isoantigenic complex CDEFK while A.SW contains the H-2-group CSEFG. Provided that the original MSWB line contains the complete isoantigenic set of both parental strains, it would thus be necessary that the variants have lost at least 2 components, namely D and K. Serological examination has shown that while the original line certainly contained D and K, both antigens were regularly absent from three independent variants, selected for growth in A.SW (3). Subsequent to this finding, the question arose whether it would be possible to select variants that have lost only one of the two isoantigenic components. As suitable hosts for such experiments A.SW × DBA and A.SW × C3H F_1 hybrids were chosen. The isoantigenic composition of A × A.SW F_1 hybrids determined by H-2 is $\frac{\text{CDEFK}}{\text{CEFGS}}$, the corresponding situation for A.SW × DBA F_1 would be $\frac{\text{CEFGS}}{\text{CDE'F}}$ and for A.SW × C3H F_1 it would be $\frac{\text{CEFGS}}{\text{CEK}}$. Upon inoculation of the MSWB tumor of the original line, A.SW × DBA mice would therefore respond with anti-K and A.SW × C3H mice with anti-D antibodies. Variants arising in such hybrids might represent monofactorial losses if specific for the hybrid in question. Testings of this type have been carried out with the MSWB sarcoma. In A.SW × DBA F_1 hybrids, progressively growing tumors were obtained in 8 out of 20 animals (40 per cent) while the corresponding figure was 19/50 for A.SW × C3H F_1 (38 per cent). In a A.SW itself the percentage of progressively growing tumors is usually of the order of 25 per cent [8, 12]

Three tumors appearing in inoculated A.SW × DBA F_1 hosts were tested further They have been carried for 3, 7 and 17 passages in A.SW × DBA F_1 mice, resp They grew progressively in 100 per cent of A.SW × DBA F_1 mice (49/49) even if the animals were preimmunized against tissue of strain A origin or against the original line of the MSWB tumor (12/12) In A.SW × C3H F_1 they did not grow better and grew possibly less well than the original MSWB tumor (17/61 28 per cent) Although there are no serological tests available on this material as yet the transplantation results would tend to indicate that the A.SW × DBA variants do not contain component K in active form but have still preserved component D they are certainly distinctly different from the variants selected in A.SW

One of the A.SW × DBA F_1 variants was subsequently adapted to A.SW × C3H F_1 hosts also After two passages in this genotype it grew in 9/9 A.SW × C3H F_1 mice In A.SW × C3H preimmunized against strain A tissue it grew in 50 per cent (8/16) When it was carried through A.SW × C3H for 3 passages and tested subsequently the following data were obtained A.SW × C3H F_1 9/9 the same preimmunized against A tissue 18/19 A SW preimmunized against A tissue 3/3 Meanwhile this line maintained its ability to grow in 100 per cent of preimmunized A.SW × DBA mice. Speaking in serological terms these data indicate that variants which have lost K but not D can be made to loose D also if carried successfully through A.SW × DBA and A.SW × C3H mice in succession

The behavior of the tumors appearing in A.SW × C3H after the inoculation of the original unselected MSWB sarcoma was different from the *A.SW × DBA* variants and more complicated Eleven such tumors were tested *taken directly after one passage in A.SW × C3H F_1* hosts The pooled results show that they grew in 64 per cent (53/82) of A.SW × C3H mice on subsequent testing, and in 29 per cent (24/84) if the hosts were preimmunized against tissue of strain A origin It has to be pointed out that there was considerable variation between the individual tumors in this respect, and 6 tumors grew in all untreated A.SW × C3H and 2 also in all preimmunized hosts. (It may be worth *mentioning in this connection that the original MSWB tumor did not* grew in 100 per cent of untreated A.SW × DBA F_1 hybrids and in a F_1 mice.) Surprisingly enough five tumors taken from A.SW × C3H grew in 100 per cent of untreated A.SW × DBA F_1 hybrids and in a variable but fairly high proportion of preimmunized A.SW × DBA (75 per cent on an average) The same five variants grew in 100 per cent of untreated and 30 per cent of preimmunized A.SW × C3H

Seven A.SW × C3H variants were tested after two consecutive passages in this genotype. As compared to the results of the first passage they showed an improved ability to grow in this genotype upon subsequent testing (39/43 91 per cent in untreated and 49/67 73 per cent in preimmunized A.SW × C3H mice). Four of the seven lines grew in 100 per cent of preimmunized A.SW × C3H.

Interpreted in isoantigenic terms these data suggest that component D cannot be lost from this tumor without K also being lost simultaneously and that the loss of both components is an event which is less likely and/or it occurs in a larger number of steps than the loss of K only in A.SW × DBA. Experiments are now in progress to find out whether this difference is peculiar to the particular tumor used or has more general validity. Because of the possibility of direct isoantigenic analysis of the tumor cells by means of cytotoxic tests[7] lymphomas originating in A × A.SW F_1 hybrids are used as the material of choice for these studies.

These results clearly indicate that different kinds of variants may be selected from one and the same tumor-cell population and that some of them may represent the end result of more than one change in relation to the original cell type. The latter point is also emphasized by the previously mentioned finding that parental variants of some F_1 tumors grow at first only in non-immunized hosts and additional selective transfer in the same strain is necessary to bring about growth in preimmunized hosts. As pointed out by Mitchison[21] and also indicated by some of our results coisogenic resistant lines may differ from each other by more than one histocompatibility factor and several successive steps of change may therefore be required before a variant can become fully compatible with a preimmunized parent. It is well known[1, 21] that preimmunization is useful for demonstrating the existence of minor isoantigenic differences between tumor and host, not revealed by simple transplantation tests.

It may be of interest to point out again in this connection that while it was possible to obtain variants specific for hosts that *lack* some H-2-determined isoantigenic components in relation to the A × A.SW F_1 hybrid of origin (such as A.SW, A.SW × DBA F_1 and (with some tumors, although not with MSWB) in A, no variants could be extracted that would have been specifically and selectively compatible with a host that contains H-2-isoantigens not possessed by the genotype of origin such as A × A.BY, A × A.CA, A.SW × A.BY or A.SW × A.CA F_1 hybrids. Of course, all variants compatible with any of the parental strains grew also in all F_1 hybrids where the same parental strain entered the cross but they were not specifically compatible with any

ginal line of the MSWB tumor in A.SW × C3H mice, pretreated with heavily irradiated cells[11] and was found to contain isoantigenic components derived from the A strain and not present in A.SW × C3H this was probably the reason why it did not grow in preimmunized A.SW × C3H mice. Nevertheless, its ability to grow in all non immunized mice of this genotype is a distinct change as compared to the original MSWB tumor. This change has occurred during a single passage in A.SW × C3H and could not be reversed by 10 passages in A × A.SW.

Another independent line has been started from the variant described in the previous paragraph the only difference being that this line has been carried for one additional transfer generation in A.SW × C3H. When tested immediately thereafter it grew in 3/3 non immunized and 3/3 preimmunized A.SW × C3H mice. Subsequently this line was also carried for 10 consecutive transfers in A × A.SW F_1 mice. Following this procedure it grew progressively in 14/14 non immunized and 28/30 (93 per cent) preimmunized A.SW × C3H mice. It still seemed to contain some antigens derived from the A strain and absent from A.SW × C3H when tested by irradiated cells. This antigenicity disappeared completely however after one more passage through A.SW × C3H hosts.

In summary these experiments indicate that the development of variants is a process that cannot be readily reversed by returning the tumor to its original genotype and maintaining it there during a series of passages. They illustrate again the point that the ability of a variant to grow in preimmunized mice may develop gradually in two or more steps.

5. « Recombination » experiments

In order to detect the possible occurrence of genetic recombination between different tumor cells, highly specific tumors (i.e. tumors showing little or no tendency to give rise to variants spontaneously) containing different H-2 markers were mixed in approximately equal amounts. Usually a sarcoma of A × A.SW F_1 origin (H-2^a/H-2^s) was mixed with an A.BY × A.CA F_1 sarcoma (H-2^b/H-2^f). The mixture was inoculated into newborn DBA/2 (H-2^d/H-2^d) or C3H (H-2^k/H-2^k) mice subcutaneously. These hosts were chosen with the intention to use immature animals as inactive as possible with regard to their homograft reaction. Since their H-2-constitution was different from both hybrid tumors and their possible recombinants it was hoped that negative selective pressures against the latter might be avoided or minimized in this way. The mixed inocula developed into tumors in nearly 100 per cent of the newborn mice after approximately two weeks. The tumors were tested in

the original A × A.SW and A.BY × A.CA F_1 hybrid types. Both components of the original mixture could be recovered and subsequent tests showed that one passage through the genotype of origin was usually sufficient to separate the original tumors in pure form. There was no evidence for any change in specificity due to this procedure, and the component tumors did not seem to develop an increased ability to give rise to variants in the parental strains.

The mixed tumors removed from the infantile DBA or C3H mice were also tested for possible specific recombinants selectively compatible with F_1 hybrids of the types A × A.BY (H-2^a H-2^b), A × A.CA (H-2^a H-2^f), A.SW × A.BY (H-2 H-2^b) and A.SW × A.CA (H-2 H-2^f). A few progressively growing tumors were obtained in some cases but these turned out to be « false positives » incapable of continued growth in the same genotype on serial transfer or variants of the conventional type, compatible not only with a new F_1 hybrid type but also with one of the parental strains of the F_1 and all other F_1 combinations where the same parent has entered the cross. No clear-cut evidence could be obtained to show the occurrence of genetic recombination since no new types were obtained that would have been specifically and exclusively compatible with a new F_1 hybrid combination. However negative experiments do not exclude the possibility of recombination and this work is therefore being continued under varied experimental conditions and with different tumors.

6. *Discussion on the possible nature of variant formation*

Since there are no direct methods available for studies on the genetic constitution of normal or neoplastic somatic cells, this discussion must remain indirect and rather speculative. The phenomena observed can be considered from various angles. According to one possibility the origin of variants compatible with the parental strains might be analogous with certain other known phenomena, essentially due to the inductive action of some factor in the new host environment. In particular « immunological enhancement » by antiserum [10] and the induced adaptation of Barrett and Deringer [2, 11] may be considered. The former phenomenon, details of which have been recently reviewed by Kaliss [10] is essentially the growth of otherwise specific tumors in certain foreign hosts that have been pretreated with lyophilized tissues of the same H-2 constitution as the tumor or with antisera directed against the H-2 components differentiating the tumor from the host. The presence of the antiserum can be effected « either by active immunization with tissues from the strain of mice to which the test tumor is indigen-

ous or by passive immunization with hetero- or isoantisera »[1] The modus operandi of immunological enhancement has been pinpointed as the exposure of the tumor graft to specific humoral antibody According to one hypothesis, enhancement is due to some « physiological » alteration in the tumor induced by its contact with antiserum which insures its survival despite the hostile responses of the host When enhanced tumors are tested in mice preimmunized by a non-enhanced tumor they usually fail to grow at least when the time interval between the immunizing (first) inoculum and the grafting of the enhanced tumor is short enough (e g 7 days). Enhanced tumors were found to grow for a short number of serial passages in untreated mice of the foreign strain concerned but died out eventually after a variable number of transfers. All published experiments with enhanced tumors deal with the growth of tumors derived from homozygous strains in other homozygotes of foreign strains.

It might be speculated that our « variant formation » could be due to the contact of the tumor cells with antibody and subsequent immunological enhancement. There are several reasons, however that make this explanation rather unlikely Variants « breed true » and can be maintained in their new hosts indefinitely They do not change back to the original condition when returned to the original genotype Once established their growth cannot be prevented by the use of hyperimmunized hosts In order to avoid the potential source of error that might arise as a result of secondary enhancement in immunized hosts we have performed control tests on a representative collection of *variants. in mice preimmunized 7 days before the test inoculation the variants grew in such animals as well as in those where the interval* between the immunizing challenge and the actual testing was longer If « variant formation would be due to enhancement there is no reason why variants » could not be obtained from homozygous tumors In the case of heterozygous tumors enhancement would be equally probable to occur in other F_1 combinations or in homozygous strains other than the parental ones *On the other hand our « false positives » can* be obtained from homozygous tumors and also from heterozygous tumors in genotypes outside the parental types they fail to breed true on repeated testing and can be prevented to a certain extent at least by the use of preimmunized mice They are more likely to belong to the same category of phenomena as « immunological enhancement

In order to test whether enhancement by antiserum treatment can lead to the establishment of « variants » in our system experiments have been started to test the behavior of F_1 tumors growing in enhanced mice of one of the parental strain Our experience is limited

to two tumor-host combinations as yet in these cases growth could be obtained in the parental mice pretreated with antiserum, but the « enhanced » tumors failed to breed true on repeated testing in untreated mice of the same strain and would therefore classify as « false positives » rather than « variants »

Although variant formation thus appears to be a phenomenon different from immunological enhancement, it does not necessarily follow that antiserum or other influences in the parental host are of no importance for its mechanism. Another case of tumor modification has been described[2, 14] that is clearly due to an interaction between some isoantigenic system(s) of a homozygous tumor (probably not the H-2 system) and a heterozygous compatible F_1 host This modification is not due to selection and it brings about a permanent specific alteration of the tumor A parallel has been drawn between this change and certain serotype transformations in ciliate protozoa[28] The present instance of variant formation is not unlike another observation made on Paramecia by Sonneborn Schneller and Craig viz. the behavior of certain serotype alleles, both of which are expressed in heterozygotes[28] Loss of the phenotype corresponding to one allele was repeatedly observed this was not due to total loss of the corresponding allele, however, since the missing phenotype could be restored by change of temperature. The findings were interpreted as being due to *differential expression* of alleles in heterozygotes. Homozygotes for serotype genes and for other genes did not show this variation. By analogy it might be speculated that exposure of heterozygous tumor cells to the environment of one parental strain may result in the differential suppression of the isoantigenic products of the H 2 allele derived from the opposite parental type, due to the action of antibody or by other means. Experiments are now in progress to investigate the possibility of restoring the missing antigens by various manipulations in vitro and in vivo

Meanwhile other explanations are by no means excluded Ambiguities of interpretation are due to the difficulties involved in all attempts to distinguish between genotype and phenotype in somatic cells. Host-induced modifications at the genetic level would include phenomena analogous to transduction involving the incorporation of genetic material from the host or non-specific increase in genetic variation provoked by some factor involved in the homograft response of the parental strain. There is no binding evidence available for the occurrence of the latter phenomenon as yet the former can be excluded by the lack of variants that would be specific for other F_1 types and contain one H 2 allele foreign to the tumor In the case of transduction

this occurrence would be no less likely than the appearance of variants compatible with the parental types.

We may now consider the *arguments that would favour* an « immunoselection »[4] of isoantigenic cell variants preexistent in the F_1 tumor prior to testing in the parental strains. The parental environment would have a purely selective effect in this case in analogy with the model experiments with artificial mixtures[13]. This explanation has been considered previously[3, 13, 15] and was regarded as fairly probable for the following reasons.

Chromosomal analysis of variants obtained from the MSWB and MACC sarcomas revealed that different variants arising *from the same* tumor at different times in the same parental strain have an individually characteristic chromosomal constitution that differentiate them from each other and from *the original line*[3]. In one instance two variants that arose in two different ASW hosts after the inoculation of two different aliquots of the same pool of tumor cells, *derived directly from* the original line of the MSWB sarcoma (maintained in A×ASW F_1 hybrids) had exactly the same chromosome constitution. This evidence tends to indicate that here we are dealing with the repeated selection of a single variant clone. In another case the appearance of a variant compatible with the parental A strain and showing chromosomal changes could be traced within the original unselected population of the MACC sarcoma[8]. Suggestive as they are, these *findings* are nevertheless only of indirect value, since the frequency of variants was not appreciably *reduced* when very small cell doses were inoculated into the parental strains, thus minimizing the importance of selecting preformed variant clones[3, 14]. Also cases of variant formation have been recently found by K. E. Hellström[8] where a chromosomal change does not seem to accompany the establishment of variant lines.

If it were assumed that variant formation is due to random changes at the genetic level, various mechanisms might be discussed, such as point mutations, more extensive chromosomal changes, somatic segregation, etc. The likelihood of point mutation could be questioned since several isoantigens are *usually lost simultaneously, such as D and* K, which are now believed to be parts of a complex pseudoallelic system, *and* crossing over has been demonstrated to occur between them[9]. Also, if point mutations were responsible, it is difficult to see why some mutants compatible with new F_1 hybrid combinations should not appear. More extensive chromosomal rearrangements have been actually demonstrated in connection with some types of variant formation. Such rearrangements may involve the H-2 locus or, *by changing the genetic* background, change the mutability and/or expression of H-2 determined

isoantigenic systems. The general validity of chromosomal changes may be questioned, however on basis of the above mentioned recent findings of Hellström [8]. Finally somatic segregation may be assumed as a possible mechanism, but its likelihood can be questioned because the frequency of the two complementary types is seldom equal and may show very considerable differences. The latter argument is not entirely conclusive, however since additional non H-2-differences between IR lines may interfere with the manifestation of one of the two complementary types.

Thus while the appearance of specific variants compatible with one of the parental strains seems to be a phenomenon limited to heterozygous F_1 tumors and is distinctly different from the development of « false positives » growing in spite of a homograft reaction and from immunological enhancement, the details of the cellular mechanism underlying the change and its genotypic or phenotypic nature remain to be investigated.

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DISCUSSION

GORER Antigenic loss can be demonstrated in homozygous tumours by absorption experiments. We had an A-strain tumour that lost its D component but showed an increased power to absorb anti K When Dr Kaliss brought Sa I to Guy's Hospital it lost E and some power to absorb anti F and anti K but D appeared unchanged. Amos found that these changes had not occurred in the Bar Harbor Sa I

KLEIN Our experiments do not in any way exclude the possibility that antigenic losses may occur in homozygous tumours, but it seems to be difficult or impossible to select variants from them that would be specifically compatible with a new homozygous strain This would require the loss of all specific H 2 isoantigens, controlled by both alleles in a diploid cell.

MITCHISON Do you find that the ASW strain exhibits more hybrid vigour and variation in susceptibility to tumour isografts than the A strain ? Do you think that variability of this sort could account for some of the preferences of the heterozygous tumours ?

Could you tell us more about your attempts to recover the expression of antigens lost in variant tumours ? Why do you think treatment with antibody *in vitro* might work ?

KLEIN *ASW does not seem to differ from A with regard to* vigour as judged by litter size, infantile mortality and growth rate. Our *attempts to « recover » the missing phenotype have so far not given any* positive results they are based on the speculation that variant formation might be due to the suppression of the expression of one of the two alleles e g at the cell-surface level If so suppression of the remaining isoantigens by antiserum treatment *in vitro* or attempts to re-select in the opposite parental genotype might result in the re-establishment of the missing phenotype We have at present no evidence that this can actually occur

OWEN The possibility that a system analogous to « serotype transformation » may be involved in transplantation antigens suggested in Dr Klein's discussion though regarded as less probable as an explanation for his phenomenon than is genetic change nev...
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tolerance not only to the antigens *expressed* in cells at any given time, but also to antigens which the living cells have the *potentiality* of developing in serotype-transformation ? If this is so tolerance for living cells imparted by inanimate antigens would seem an almost hopeless prospect, perhaps approachable only by using mixtures of antigens from a variety of sources. Second could it be that the possible relative « non specificity » of tolerance as compared with immunization for second-set responses, which I suggested in the discussion of Dr Hašek's paper also relates to a serotype transformation system ? Pre immunization against the antigens *expressed* in an immunizing tissue may provide a much narrower base for cross-immunization than conferring tolerance against all the antigens that may be potential to a living tissue established in a very young recipient.

Medawar Dr Klein would you remind us of the technique of pre-immunization which you use to sharpen your immunological discrimination ? Pre-immunization is a good deal more complex than we originally thought, because of the intervention of enhancement difficulties. Pre immunization by skin homografts is semi-permanent and seems to be free from these particular difficulties

Klein In order to avoid the difficulties arising from secondary enhancement, we have tested a representative number of variants in mice immunized 7 days previously by injections of spleen, or mixed liver and spleen. At this stage, secondary enhancement would not yet be expected to occur Variants compatible with mice immunized 20-30 days previous to injection also grew regularly in 7-day immunized animals.

DISCUSSION

GORER Antigenic loss can be demonstrated in homozygous tumours by absorption experiments. We had an A-strain tumour that lost its D component but showed an increased power to absorb anti K. When Dr Kaliss brought Sa 1 to Guy's Hospital it lost E and some power to absorb anti F and anti K but D appeared unchanged Amos found that these changes had not occurred in the Bar Harbor Sa 1

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OWEN The possibility that a system analogous to « serotype transformation » may be involved in transplantation antigens suggested in Dr Klein's discussion though regarded as less probable as an explanation for his phenomenon than is genetic change, nevertheless promotes some interesting speculations. I would like to mention two first could it be that failure to confer tolerance to tissue transplants by means of killed cells or cell-free preparations relates to the necessity of conferring

tolerance not only to the antigens *expressed* in cells at any given time but also to antigens which the living cells have the *potentiality* of deve lop[ing] ; in serotype transformation ? If this is so tolerance for living cells imparted by inanimate antigens would seem an almost hopeless prospect, perhaps approachable only by using mixtures of antigens from a variety of sources. Second could it be that the possible relative « non specificity » of tolerance as compared with immunization for second-set responses which I suggested in the discussion of Dr Hašek s paper also relates to a serotype-transformation system ? Pre immunization against the antigens *expressed* in an immunizing tissue may provide a much narrower base for cross-immunization than conferring tolerance against all the antigens that may be potential to a living tissue esta blished in a very young recipient.

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Immunisation de souris C57BL par un *sarcome spontané* provenant d une souris de même race

E. H Betz (*)

Laboratoire d Anatomie Pathologique de l'Université de Liège

La question de savoir si les tissus *tumoraux possèdent des propriétés* antigéniques différentes de celles des tissus normaux de l organisme a déjà fait l objet de nombreuses études. Jusqu à ces dernières années pourtant les résultats publiés n étaient guère convaincants on en trouvera la discussion dans une revue générale publiée par Hauschka [18] en 1952.

Plusieurs auteurs ont suggéré que les cellules tumorales peuvent etre dépourvues de certains antigènes présents dans les cellules normales dont elles dérivent On en est même arrivé à considérer que cette « perte des antigènes » est une des caractéristiques des tissus tumoraux (Green [13]) En fait le problème semble plus complexe et des recherches récentes ont montré que les cellules tumorales ne se caractérisent pas nécessairement par une perte de certaines de leurs propriétés antigéniques mais peuvent posséder des antigènes différents de ceux des cellules normales. La démonstration de ces différences antigéniques n est pas toujours facile à réaliser Nous nous intéresserons uniquement ici aux résultats obtenus par les auteurs qui ont étudié des tumeurs expérimentales non filtrables. *Dans le cas des tumeurs filtrables, en effet* le *problème devient* beaucoup plus complexe encore.

On trouve dans la littérature quelques observations ou des animaux de lignées apparemment pures ont été immunisés contre des tumeurs apparues chez des sujets de meme lignée (Aptekman et coll [1] Goldfeder [12] Gross [17] McDowell et coll [21]). D autres recherches encore confirment l existence d une modification antigénique des cellules tumorales. Des expériences de Kidd [23] sur la tumeur de Brown Pearce plaident en faveur de cette hypothèse Il en va de meme pour certaines expériences de Green [14] et de Imagawa et coll [22] qui comparèrent l action cytotoxique sur les cellules tumorales du sérum provenant d animaux

(*) Associé du Fonds National de la Recherche Scientifique

immunisés soit par du tissu normal soit par du tissu tumoral ces auteurs auraient observé des différences d'activité assez nettes Foley [12] réussit à immuniser des souris C_3H contre des sarcomes provoqués par le méthylcholanthrène chez des sujets de même lignée. Prince et coll [illegible] obtinrent récemment des résultats analogues en immunisant des souris D.B.A. par des suspensions de cellules tumorales provenant d'animaux de la même lignée.

Enfin de nombreux travaux ont été consacrés à l'étude des réactions immunologiques comparatives chez les animaux injectés de préparations antigéniques diverses isolées à partir de tissus normaux et de tissus tumoraux. Pour plus de détails à ce sujet nous renverrons à la revue récente publiée par Zilber [illegible]

Avec G Desoignies [illegible] nous avons eu l'occasion d'isoler chez une souris C57BL un sarcome spontané que nous avons appelé « sarcome J ». Ce sarcome polymorphe est aisément greffable et a la propriété de présenter des régressions spontanées quand il est greffé à des souris homozygotes de la même lignée C57BL. Cette tumeur spontanée de comportement assez exceptionnel nous a paru un matériel convenant particulièrement pour l'étude des modifications antigéniques survenant eventuellement dans les tissus tumoraux. Nous résumerons ici quelques-unes de nos observations sur le comportement de cette tumeur au cours de transplantations successives.

1 — *Croissance du sarcome J au cours de transplantations successives*

Pour apprécier la croissance de cette tumeur nous avons utilisé une méthode que nous avons décrite précédemment. Elle consiste à mesurer régulièrement le plus grand et le plus petit diamètre des tumeurs greffées dans le tissu sous-cutané. Il est possible de calculer alors le diamètre moyen correspondant au diamètre d'une sphère de même volume que la tumeur Au cours des 15 premiers jours qui suivent la greffe, le diamètre moyen augmente de façon linéaire. Le coefficient angulaire de cette droite peut être considéré comme le coefficient de croissance. Pour chaque tumeur on peut determiner ainsi le coefficient de croissance individuel et pour un lot d'animaux établir un coefficient de croissance moyen.

Si on fait ces déterminations au cours de 146 transplantations successives, on obtient les résultats suivants

Coefficient de croissance à la 5e transplantation	} [illegible]
Coefficient de croissance à la 11e transplantation	
Coefficient de croissance à la 25e transplantation	0,72
Coefficient de croissance à la 62e transplantation	0,74
Coefficient de croissance à la 95e transplantation	0,78
Coefficient de croissance à la 140e transplantation	0,75
	0,77

On voit que la tumeur a poussé plus rapidement au cours des premières transplantations, mais à la 11 déjà elle a acquis une vitesse de croissance stable qui est restée inchangée à la 140° transplantation. La fréquence des régressions spontanées n'a pas non plus présenté de variation considérable une fois que la croissance a été stabilisée. Alors qu'à la 5° transplantation 5 % des souris présentaient une régression spontanée de la tumeur la fréquence des régressions s'est maintenue ultérieurement aux environs de 30 % pour des lots mixtes comprenant des souris des deux sexes. Toutefois, nous avons constaté que la fréquence des régressions spontanées varie considérablement avec le sexe de l'hôte inoculé

2 — *Influence de la race et du sexe sur la croissance et la régression du sarcome J*

Nous avons étudié la fréquence des régressions spontanées sur des souris C57 des deux sexes ainsi que sur des souris appartenant à d'autres lignées pures C3H et R3A. Nous avons également greffé la tumeur chez des hybrides F1 de C57 et C3H et des hybrides F1 de C57 et R3A. Le tableau I donne le pourcentage des régressions spontanées apparues dans les différents lots d'animaux greffés du sarcome J Il ressort très nettement de ces résultats que la race et le sexe influencent considérablement le nombre des régressions spontanées.

TABLEAU I — *Fréquence des régressions spontanées du sarcome J chez des souris de races et de sexes différents*

Race.	*Mâles*	*Femelles*
C57	19,4 % ± 5,6	40,8 % ± 6,2
C3H	60 % ± 7	63 % ± 7,2
R3A	33,3 % ± 6,9	60,4 % ± 6,7
F_1 (C57 ♀ × C3H ♂)	0	0
F_1 (C3H ♀ × C57 ♂)	0	0
F_1 (C57 ♀ × R3A ♂)	0	0

Alors que les souris C3H présentent une proportion élevée de régressions identiques dans les deux sexes les souris *C57* et R3A *montrent une* nette différence sexuelle. Dans ces deux dernières races, des femelles ont présenté deux fois plus de régressions spontanées que les mâles. Par

contre le chiffre absolu des régressions est très différent d'une lignée de souris à l'autre. Chez les souris R3A, nous avons obtenu plus de régressions que chez les animaux C57 du sexe correspondant. Enfin chez les hybrides F1 provenant du croisement de C57 et de C3H ou de C57 et de R3A nous n'avons jamais observé de régressions spontanées alors que les deux souches dont ces hybrides dérivent en présentent une proportion notable.

3. — *Influence de l'endroit d'inoculation sur les régressions spontanées du sarcome J*

Les expériences dont il vient d'être question ont été réalisées sur des tumeurs implantées dans le tissu sous-cutané. Nous avons greffé le sarcome J à trois lots comprenant chacun 40 souris femelles C57BL. Les greffes furent faites respectivement par voie sous-cutanée, intramusculaire et intrapéritonéale. Les souris recevant une greffe intrapéritonéale succombent rapidement à leur tumeur puisque tous les sujets de ce lot sont morts 17 jours après la greffe. Lorsque l'implantation du greffon est faite dans les muscles de la cuisse, tous les sujets ont succombé au 24e jour de l'expérience. Par contre, l'implantation sous-cutanée de la tumeur entraîne une mort plus tardive et dans 38 % des cas nous avons observé une régression spontanée de la masse tumorale avec survie de l'animal. On voit donc que les régressions spontanées n'apparaissent que chez les souris inoculées de tumeur dans le tissu sous-cutané.

4. — *Evolution de l'immunité après régression spontanée*

Le sarcome J lorsqu'il a régressé immunise l'hôte contre une nouvelle inoculation du même tissu tumoral. Nos recherches ont porté sur 232 souris C57BL qui ont survécu à une première greffe de sarcome J. Ces souris ont présenté une tumeur qui, après une période de croissance normale, a spontanément guéri. Un à 8 mois après la guérison de cette première tumeur nous avons réinoculé les souris avec le même sarcome J et nous avons suivi la croissance du greffon.

Le comportement de ce deuxième greffon varie suivant les cas. On peut distinguer quatre types de réponses différents :

a) il peut se faire que le greffon tumoral ne se développe pas et soit complètement résorbé ; *b*) il arrive aussi que le greffon augmente légèrement de volume pour donner naissance à un petit nodule tumoral de 3 à 5 mm de diamètre ; ce nodule reste stationnaire pendant plusieurs jours et finalement se résorbe ; *c*) d'autres fois encore, le greffon subit un développement lent et progressif pendant 2 à 3 semaines, après quoi la tumeur diminue de volume, s'ulcère et s'élimine spontanément ;

d) enfin dans une minorité de cas, nous avons observé un développement normal du greffon aboutissant à une tumeur mortelle

L'importance relative de ces quatre types de réponse varie en fonction du temps qui sépare la régression de la première tumeur de la deuxième inoculation. Dans le tableau II nous donnons les proportions des divers types de réponse en fonction de ce délai.

TABLEAU II — *Croissance du greffon chez des souris immunisées en fonction du délai séparant la greffe d'une première régression*

Délai après 1re régression	*Nombre de souris*	*Pas de prise de greffon.*	*Petite tumeur stationnaire puis régression.*	*Tumeur à croissance ralentie puis régression.*	*Tumeur mortelle*
1 mois	58	56 (96,5 %)	2 (3,5 %)	—	—
2 mois	46	25 (54,3 %)	16 (34,8 %)	5 (10,9 %)	—
3 mois	30	16 (53,3 %)	11 (36,6 %)	3 (10 ″)	—
4 mois	28	6 (21 4 %)	11 (39,3 %)	11 (39,3 %)	—
5 mois	47	2 (4,2 %)	20 (42,5 ″)	22 (47,2 %)	3 (6,3 %)
8 mois	21	2 (9,5 %)	4 (19 ″)	9 (42,8 ″)	6 (28,6 ″)

On y voit que chez des souris recevant la 2 inoculation un mois seulement après la *régression de la première tumeur la grande majorité* des greffons a été résorbée sans montrer le moindre signe de croissance. Au fur et à mesure que le délai séparant la régression de la 2 implantation tumorale augmente, la proportion des greffons capables de se développer augmente également. Ces greffons donnent de plus en plus fréquemment de petits nodules stationnaires, puis plus tard encore des tumeurs à croissance ralentie. Enfin 5 à 8 mois après la première régression un certain nombre de tumeurs apparaissent qui sont capables de tuer le porteur

5 — *Influence de l'immunité sur la croissance d'autres tumeurs de la souris C 57*

Nous avons essayé de voir si l'immunité consécutive à la régression spontanée du sarcome J modifie la croissance ultérieure d'autres tumeurs *de la souris C57BL. Pour cela nous avons greffé à des souris immunisées* deux autres souches de sarcome spontané isolées à partir de souris C57Bl du même élevage et un fibrosarcome expérimental produit par du méthylcholanthrène. Dans aucun cas nous n'avons observé de modification de la croissance de ces nouvelles tumeurs Celles-ci entraînent infailliblement la mort du porteur L'immunité conférée par la régression du sarcome J paraît donc très spécifique

6. — *Evolution de l'immunité au cours de la croissance tumorale*

Avec G Booz, nous avons tenté de déterminer la façon dont évolue l'immunisation au cours même de la croissance du sarcome J Pour cela nous avons greffé ce sarcome à des souris C57BL et après un délai de 6 ou 10 jours nous avons enlevé chirurgicalement la tumeur Lorsque la plaie a été cicatrisée, nous avons procédé à une nouvelle greffe du sarcome J et avons étudié la croissance de ce second greffon.

Sur 12 animaux qui ont été porteurs d'un premier sarcome J pendant six jours, la deuxième greffe a poussé normalement dans 8 cas. Elle a régressé dans 2 cas et le greffon n'a pas pris dans 2 autres cas.

Sur 44 souris qui avaient porté leur première tumeur pendant 10 jours, la deuxième greffe tumorale n'a pas pris dans 40 cas elle a poussé normalement chez 4 souris seulement. Ces résultats encore fragmentaires montrent que l'immunité apparaît après un certain délai et qu'après 10 jours de croissance tumorale la plupart des sujets ont été immunisés contre le sarcome J

— *Influence de la castration et du benzoate d'œstradiol sur les régressions spontanées du sarcome J greffé à des souris mâles*

Nous avons montré plus haut que le pourcentage des régressions spontanées est notablement plus élevé chez les femelles que chez les mâles de race C57BL et R3A. Nous avons étudié d'abord les modifications des régressions spontanées chez des animaux castrés appartenant à ces deux races. Le tableau III montre que chez les souris mâles la castration préalable entraîne une augmentation significative du nombre des régressions spontanées. Celles-ci deviennent à peu près aussi fréquentes chez le mâle castré que chez la femelle normale. L'augmentation du pourcentage des régressions spontanées chez le mâle castré s'observe dans les deux races de souris que nous avons étudiées

TABLEAU III — *Fréquence des régressions spontanées chez les souris castrées*

Race	Mâles	
	Normaux	Castrés.
C57	20 % ± 6.7	59 % ± 5.8
R3A	43.5 % ± 6.3	64.2 % ± 6.5

Chez des souris mâles de race C57BL, nous avons étudié également l'influence du benzoate d'œstradiol sur la fréquence des régressions spontanées. Ce benzoate d'œstradiol est injecté à raison de 5 ou 10 microgrammes/jour à partir du moment de la greffe. Les résultats fournis par cette expérience furent les suivants :

C57BL, mâles témoins 25 % ± 3,8

C57BL, mâles injectés de 5 μg de benzoate d'œstradiol 75 % ± 6,1

C57BL, mâles injectés de 10 μg de benzoate d'œstradiol 42 % ± 5,5

On voit ainsi que les injections de benzoate d'œstradiol permettent d'augmenter considérablement la fréquence des régressions spontanées chez le mâle et d'obtenir même des pourcentages de régression supérieurs à ceux observés chez les femelles normales.

8 — *Influence de la cortisone sur la croissance et les régressions spontanées du sarcome J*

On sait que l'administration de cortisone permet de modifier considérablement les réactions immunologiques. Avec Dewart et Piette, nous avons recherché quelles seraient les répercussions d'un traitement de ce genre sur la croissance et la régression spontanée du sarcome J. Pour cette étude, nous avons utilisé trois lots différents de souris C57BL mâles. Le premier de ces lots a été injecté journellement de 0,25 mg d'acétate de cortisone et cela pendant les huit jours qui précèdent la greffe tumorale. Après cette dernière, le traitement hormonal a été définitivement arrêté. Le deuxième lot reçoit la même dose journalière de cortisone pendant les six jours qui suivent la greffe de la tumeur, c'est-à-dire avant que la croissance tumorale ne soit macroscopiquement perceptible. Le troisième lot est injecté de la même façon, mais le traitement n'a été instauré qu'à partir du 6ᵉ jour qui suit la greffe et a été poursuivi jusqu'à la mort de l'animal ou jusqu'au moment où la tumeur a complètement régressé. Dans cette dernière expérience, la cortisone a donc été administrée pendant la période de croissance de la tumeur.

Les résultats de ces recherches ont été réunis dans le tableau IV. On constate que sous l'influence du traitement hormonal, la croissance du sarcome J peut être nettement modifiée. Cette modification dépend du moment où le traitement par la cortisone a été instauré. Lorsque les souris sont traitées avant la greffe, la vitesse de croissance est nettement accélérée. Si la cortisone est injectée pendant les six jours qui suivent la greffe, la croissance n'est guère modifiée. Enfin, si l'administration d'hor

mones est faite à partir du 6 jour qui suit la greffe la croissance tumorale est nettement ralentie.

TABLEAU IV — *Coefficients de croissance et fréquences de régressions spontanées chez les souris C57 mâles traitées par la cortisone*

	Coefficient de croissance	Régressions spontanées.
Témoins C57	0,88 ± 0,03	19,4 % ± 5,6
Injectés de cortisone avant la greffe	1,2 ± 0,04	10 % ± 3
Injectés de cortisone pendant 6 jours après la greffe	0,87 ± 0,04	6 % ± 3,1
Injectés de cortisone à partir du 6e jour après la greffe	0,72 ± 0,03	31 % ± 4,5

Le pourcentage des régressions spontanées est lui aussi influencé par le traitement hormonal. Chez les souris injectées de cortisone avant la greffe ou pendant les six jours qui suivent celle-ci le pourcentage d— régressions spontanées diminue très fortement. En effet de 19,4 % che les témoins, il passe à 10 et 6 %. Lorsque la cortisone est administrée partir du 6 jour qui suit la greffe, la proportion des régressions sponta nées est considérablement accrue et atteint 31 %

9. — *Modification du sarcome J par transplantations répétées sur hybri des F1*

Nous avons vu plus haut que le sarcome J transplanté sur des hybri des F1 de C57 et C3H croît plus rapidement que sur les races des parents et ne présente plus de régression spontanée. Nous avons effectué 72 transplantations successives sur hybrides F1 au cours desquelles la croissance tumorale est restée très stable. Après le 72 passage successif sur ces hybrides, nous avons à nouveau transplanté le sarcome sur des souris C57 et nous avons constaté que le sarcome J avait acquis des propriétés nouvelles.

Après son passage chez les souris hybrides le sarcome J présente, lorsqu'on le transplante sur des souris C57 une croissance accélérée. Cette augmentation du coefficient de croissance n'est pas transitoire, mais per dure pendant longtemps ainsi qu'en témoigne le tableau V

On y voit que la vitesse de croissance accélérée persiste après 30 transplantations successives et que cette accélération affecte tant les femelles que les mâles.

TABLEAU V — *Coefficients de croissance moyens du sarcome J adapté aux hybrides F_1 et regreffé aux souris C57*

Transplantation sur C 57 du sarcome J adapté aux souris hybrides F1	Coefficient de croissance	
	Mâles	Femelles.
1re transplantation	0,97	0,97
10e transplantation	0,96	1
20e transplantation	0,98	0,98
30e transplantation	0,99	1
60e transplantation	0,97	0,99

En outre le sarcome J modifié par des passages successifs sur hybrides F1 ne donne plus de régression spontanée lorsqu'on l'inocule aux souris C57. Au cours des 60 transplantations successives sur C57 la souche du sarcome J modifiée n'a plus donné une seule régression spontanée. Nous avons appelé Ja cette souche nouvelle dérivée de J.

Du point de vue immunologique, la souche Ja est également différente de la souche J originelle. En effet, nous avons greffé le sarcome Ja à des souris immunisées contre la souche J. Dans tous les cas, les greffons de la souche Ja ont donné naissance à des tumeurs mortelles à croissance rapide (coefficient de croissance 0,92). On voit que la souche J et la souche Ja, qui en dérive, sont devenues très différentes et que les souris immunisées contre la première ne le sont plus contre la deuxième.

Discussion

Comme nous l'avons vu dans l'introduction de ce travail, on trouve dans la littérature quelques observations où des animaux de lignée pure ont été immunisés contre des tumeurs apparues chez des sujets de même lignée. On a fait remarquer que beaucoup des observations antérieures peuvent s'expliquer par une mutation soit de la tumeur, soit de la lignée de l'hôte. Une telle interprétation est théoriquement possible lorsque les phénomènes d'immunisation apparaissent au cours d'une longue série de transplantations successives. Elle paraît néanmoins peu probable dans le cas particulier de notre observation. Nous avons vu en effet que dès la 5^e transplantation du sarcome J des régressions spontanées ont fait leur apparition. D'un autre côté l'hypothèse d'une mutation ayant atteint certains sujets de notre élevage au moment précis où nous avons entrepris d'isoler cette souche tumorale paraît hautement improbable. De

très nombreuses expériences de greffes de tissus normaux (rate, peau foie) étaient poursuivies à ce même moment sur des animaux du même élevage. Elles n'ont jamais permis de déceler des signes d'incompatibilité

L'interprétation la plus vraisemblable de nos observations est que les cellules tumorales contiennent des antigènes différents de ceux présents dans les cellules normales du même organisme. Nous avons vu que par d'autres méthodes expérimentales on arrive actuellement à la notion d'une différence de structure antigénique entre les cellules tumorales et les cellules normales.

Si le sarcome J isolé par nous possède des antigènes différents de ceux qui sont normalement présents dans les tissus de souris C57 comment expliquer que dans un même lot de souris certains sujets s'immunisent suffisamment pour guérir de leur tumeur tandis que d'autres succombent à leur néoplasme ? Nous avons montré que pratiquement toutes les souris greffées de sarcome J s'immunisent contre cette tumeur En effet, si on enlève cette dernière après 10 jours de croissance, la grande majorité des animaux est immunisée contre une greffe tumorale ultérieure. Si tous les sujets développent des anticorps il est probable qu'ils le font à des degrés très divers. On a émis l'hypothèse que des antigènes particuliers peuvent exister dans le tissu tumoral en quantités relativement réduites voisines du seuil nécessaire pour provoquer le développement d'anticorps chez l'hôte. L'immunisation de celui-ci dépendra dès lors de facteurs individuels susceptibles de favoriser ou d'inhiber la formation d'anticorps. Certaines de nos expériences montrent que l'hôte réagit différemment dans certaines conditions expérimentales et cela sans que la structure antigénique de la tumeur paraisse modifiée.

La vitesse de croissance de la tumeur doit tout d'abord être étudiée. Nous avons vu que le sarcome J inoculé sous la peau de souris C57 immunise un certain nombre de sujets. La même tumeur inoculée dans le péritoine ou les muscles tue l'hôte dans 100 % des cas. Nous avons vu qu'après une greffe intrapéritonéale ou intramusculaire, la croissance tumorale est rapide et il est vraisemblable que la tumeur tue le porteur avant qu'une immunité suffisante ait pu se développer

Un second facteur que nous discuterons brièvement, est l'hybridation Nous avons dit que chez les hybrides F1 (C57 × C3H) et (C57 × R3A) nous n'avons jamais observé de régression spontanée du sarcome J alors que les deux souches parentes en présentent des proportions notables. Des observations similaires aux nôtres avaient déjà été faites par Andervont [1] il est difficile d'en donner une interprétation satisfaisante.

Le sexe paraît jouer un rôle important dans l'immunisation de l'hôte. Nous avons montré en effet que chez les femelles les régressions

spontanées sont plus fréquentes que chez les mâles. Ceci est vrai non seulement pour les souris C57 mais aussi pour les souris R3A. Des observations analogues ont été faites par Gross [13] étudiant le sarcome 37 chez des souris albinos. Dans ce cas les souris mâles étaient plus réceptives que les femelles à l'inoculation de petites quantités de cellules tumorales et présentaient moins de regressions spontanées. Greene [14] a noté également que les lapins mâles etaient plus réceptifs que les femelles à la greffe d'une tumeur papillaire dans la chambre antérieure de l'œil.

De plus nous avons montré que, chez le mâle, le pourcentage de régressions spontanées est fortement augmenté par la castration et par l'administration d'œstrogènes. Après ces traitements la fréquence des régressions spontanées se rapproche de celle observée chez les femelles normales. Dans le même sens plaident également des observations rapportées dans la littérature qui montrent que les œstrogènes favorisent l'immunisation de souris greffées de diverses tumeurs transplantables (Nathanson et Salter [25] Salter et coll. [27] Howard et coll. [17]). D'autres auteurs encore ont signalé l'influence favorable de la grossesse sur les régressions spontanées de certaines tumeurs (Baatz [2] Emge et coll. [11] Homburger et Tregier [16]).

Plus complexes sont les résultats observés chez les souris traitées par la cortisone. Nous les avons discutes plus longuement dans un travail recent (Betz, Dewart et Piette [10]). Nous pensons qu'il faut tenir compte ici d'une double action de la cortisone. Celle-ci inhibe d'une part la croissance de la tumeur et d'autre part diminue l'immunisation de l'hôte. Cette dernière action ne se manifeste que si la cortisone est injectée avant le moment de la greffe ou immédiatement à partir de celle-ci. Nous avons vu en effet que dans ces conditions la vitesse de croissance de la tumeur augmente tandis que la fréquence des regressions spontanees diminue fortement. Au contraire lorsque le traitement cortisonique est instauré au 6e jour après la greffe, la vitesse de croissance diminue et le pourcentage des régressions augmente. Il nous a semblé qu'ici la cortisone agit directement sur le tissu cancéreux pour en freiner la croissance sans modifier l'immunisation de l'hôte.

Pour terminer nous voudrions dire quelques mots de la transformation du sarcome J par passages prolongés sur des hybrides F1 Nous avons vu qu'après 72 transplantations successives sur des hybrides, le sarcome J a acquis une croissance plus rapide et ne donne plus de regression spontanée lorsqu'on le greffe à des souris C57 Cette transformation semble etre permanente puisqu'elle persiste après 60 transplantations successives de cette nouvelle souche tumorale sur les souris C57 De plus, la nouvelle souche Ja pousse normalement chez des souris immunisees contre la souche J originelle. Des observations similaires aux

nôtres ont été réalisées par Barrett et Deringer [4] qui obtinrent des modifications d'une souche tumorale après un seul passage sur des hybrides. Les mécanismes responsables des transformations antigéniques du tissu tumoral ne sont pas encore élucidés

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DISCUSSION

Knake Dr Betz's paper is important in two respects it raises the question of tumour-specific antigens as Lumsden suggested years ago when inbred strains were not in common use. It also discusses the important problem of the change from dependent to autonomous tumours which has been discussed by Greene

Gorer I was very interested in the sex difference shown by Dr Betz. Kaliss has always stressed this. It is more difficult to enhance tumours in females than in males. However Kaliss did not succeed in showing the normal basis of the difference and Dr Betz must be con-

gratulated on doing so. From the point of view of antigenic structure, it is impossible to draw any decisive conclusions from any single tumour Dr Prehn has done a great deal of work on this subject and I would like to hear his views.

BETZ There are other examples of grafted tumours whose regression is favoured by the administration of oestrogens to the host. We are trying now to study the effect of testosterone on the behaviour of our sarcoma.

We would not dare draw any conclusion from our observations about the antigenic structure of the tumour On this subject I agree completely with Dr Gorer

PREHN In my experiments to which Dr Gorer refers a large number of methylcholanthrene induced sarcomas were tested for antigenicity within mice of their respective strains of origin Most of these were found to be antigenic. The immunity was tumour specific for the following reasons spontaneous sarcomas of the same strains were apparently not antigenic normal tissues from the same animal in which a methylcholanthrene sarcoma arose were not antigenic and normal tissue (skin) obtained from the same animal in which a tumour had arisen did not respond to the immunity induced by the tumour In my opinion without controls of this type, it is impossible to conclude that an instance of isologous tumour regression is due to tumour specific antigens. There are numerous other possible sources of variation even within strictly inbred strains of animals.

BETZ I quite agree with Dr Prehn. We only suggested that a difference in antigenic structure may explain our results but there is no proof that this is really the case. It is indeed difficult to draw inferences from a single incidental observation As we are dealing with spontaneous tumours, our observations were undoubtedly lucky for we studied various other spontaneous sarcomas in C57Bl mice and we could not find any other which behaved in the same way I would like to add that in our strain of C57 bl [illegible] mice normal tissues have been grafted from one normal anima[illegible] and these [illegible] always take. In our conditions, many [illegible] ols Dr Preh[illegible] in his experiments are impossible.

Did Dr Betz try the effect of oestrogen in the hybrid mouse which showed no regression at all and has he studied the effect of androgens on the response ?

BETZ We thought that the difference between mice grafted subcutaneously and mice grafted in other sites could be due to the fact that the animals receiving a peritoneal graft died before any effective immunity could be reached. Dr Krohn's suggestion is very attractive and deserves further study We never studied the effect of oestrogens in hybrid mice. What we did was to study the effect of adrenalectomy In C57 Black mice, adrenalectomy increases the percentage of regression In F_1 hybrids we got only two regressions after adrenalectomy this seems not to be significant

VOISIN Concerning the question of intraperitoneal route of injection and immunization I would like to ask Dr Betz two correlated questions First in the case of the intraperitoneal injection of tumour cells into non-immunized mice, did you observe any sign of immunization against a second injection made a few days after the first one, or by means of adoptive transfer ? And second, in the case of immunized mice did all the tumours later injected intraperitoneally regress ?

BETZ We never tried to graft the tumours subcutaneously into mice previously grafted intraperitoneally In fact, as the mice receiving a graft in the peritoneum die quickly it would be impossible to judge the fate of the subcutaneous graft. The only means of reaching a conclusion would be to study the adoptive transfer That we did not do

In mice immunized by a first subcutaneous graft we put a second graft intraperitoneally and that never grew

PREHN Dr Betz, I am not quite clear about one point. Your data seem to indicate that your tumour grew better in the 5th transplant generation than it did subsequently This would be a most unusual behaviour Since you have demonstrated a sex difference is it possible that the low percentage of regressions in early transplant generations was not a real biological difference but due to the chance distribution of males and females among the recipient animals used ?

BETZ That is quite possible. At the 5th transfer of our tumour we did not notice the sexual difference When we became aware of this, it was too late to have an idea of the ratio of males and females in this experimental group

Aspects biologiques des hétérogreffes artérielles et osseuses

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Introduction

En raison de leur autonomie et de leur susceptibilité très poussées les animaux supérieurs et l'Homme n'admettent théoriquement pas l'intrusion de tissus étrangers *homogènes* (donneur de la même espèce que le récepteur) et surtout *hétérogènes* (donneur d'une espèce différente que le récepteur). Aussi les transplants homogènes ou hétérogènes subissent-ils l'effet néfaste des réactions immunologiques développées par le porte-greffe et dirigées vers la dégénérescence et l'exclusion du greffon. Néanmoins l'hétérotransplantation veau/homme ou chien de greffons d'artères ou d'os conservés et dévitalisés constitue une prothèse très utilisée en chirurgie humaine du fait qu'il est possible de constituer des banques de greffons prêts à l'emploi. Cette réussite immunologique et chirurgicale dépend sans doute du traitement subi par l'hétérotransplant (formolation, congélation, cryodessiccation) peut-être débarrassé dès lors, de l'antigène générateur des réactions immunitaires.

Outre leur intérêt utilitaire les greffes artérielles et osseuses mortes évoquent le problème biologique général de l'Immunogreffologie dont l'étude débute seulement.

Définition

Dans cet exposé sera étudié l'ensemble des réactions humorales et tissulaires d'ordre immunologique développées par le porte-greffe (récepteur : chien ou homme) vis-à-vis d'un greffon hétérogène (donneur : veau) et orientées vers l'élimination de cet hétérogreffon.

Cette définition était nécessaire pour limiter le sujet et n utiliser les résultats cliniques, radiographiques histologiques des greffes que dans la mesure où ils concernent ces réactions immunitaires

Un rapide commentaire doit porter sur deux termes

« Immunité » synonyme d « incompatibilité » et d « intolerance » est un mot évoquant étymologiquement (*in munus* = exemption de charge, de sensibilité) l état privilégié d un organisme ayant developpe des substances de défense favorables, vis-à vis d un agresseur pathogène (microbe en particulier)

Ce terme, confisqué par les bactériologistes est ici impropre puisqu'il évoque les réactions de défense défavorables du porte greffe contre les tissus du greffon inséré dans un dessein curatif. On souligne ainsi le caractère aveugle de ces réactions immunitaires dénuées d intelligence et de discernement. Peut-être serait il plus conforme à la verite d adopter un terme de compréhension et d acception plus large « allergie » (*allos ergon* = état modifié d un organisme vis-à vis d un antigène)

« Greffe » dont l étymologie évoque le stylet du végetaliculteur, est un mot applicable aux seules autogreffes impliquant la survie du greffon et la fusion tissulaire intime de ce dernier et du porte-greffe C est donc par commodité de langage que ce terme est utilise ici pour l étude de transplantations d un greffon hétérologue mort dans un but de prothèse vasculaire ou osseuse.

I — L individualité d espece.

Chaque espèce d animaux superieurs adultes jouit d une individualité immunologique propre, à ce point susceptible que toute intrusion d un tissu appartenant à un individu d une espèce differente suscite des réactions de défense, et réciproquement. Par conséquent, les individus constituant chacune des espèces jouissent d une immunite mutuelle d espèce, naturelle innée, héréditaire dont la cinétique s inscrit en 3 stades

1er stade. — Immunité potentielle non révélee.

2 stade. — Immunité actualisée d'intensité moyenne sous deux déterminations possibles

— la transplantation hétérogène

— l'injection d extraits tissulaires héterogènes.

3 stade. — Hyperimmunité renforcee sous l'influence

— de plusieurs transplantations hétérogenes successives dont chacune potentialise l'immunité declenchée par la precedente

— d une transplantation héterogène unique mais precédée ou suivie d une immunisation à plusieurs reprises par des injections d ex

traits tissulaires solubles, additionnés de substances adjuvantes et stimulantes de l'immunité (corps microbiens tués par exemple)

II — Tests révélateurs des réactions immunologiques anti espèce chez le récepteur.

Dans une recherche dirigée par Wertheimer et Sautot (artères) Guilleminet et Stagnara (os) Barone Bost et Joubert (animaux) à Lyon et conduite concurremment à l'Hôpital E. Herriot et à l Ecole Vétérinaire ces tests furent choisis très simples dans leur principe et leur exécution en vue d une application pratique au contrôle pré- et post-opératoire des greffes. Ils ont concerné tout d abord les greffes aortiques chez le chien et les greffes fémorales chez l'homme puis les hétérotransplantations osseuses.

Ils appartiennent à 3 catégories

A — *Tests immunologiques proprement dits*

Ils se proposent de révéler la présence d'immunigènes dans les tissus du greffon et considèrent l ensemble de la reaction génerale et locale macroscopique et histologique, du sujet greffe Ont été considérés comme tels les documents concernant

a) l état général du sujet — toujours ébranlé en cas d echec, excellent *si la greffe réussit*

b) les examens radiologiques. Selon le type de transplant ont été interrogées

— l artériographie qui consiste à fixer sur un cliché radiographique l'image du segment vasculaire greffé, grâce à l'injection intra artérielle proximale d un produit opaque aux rayons X (diiodone) Le procédé n est d ailleurs pas sans danger et fut systématiquement proscrit chez l'homme. La réussite d une greffe se signale par un trace rectiligne de l aorte. Au contraire une dilatation ampullacée plus ou moins prononcee se remarque lorsque les parois vasculaires sont le siège de reactions immunitaires focales et cèdent progressivement sous la poussée sanguine

— l évolution radiologique des transplants osseux de grandes dimensions (pontages diaphysaires du radius chez le chien interventions prothetiques diverses chez l'homme). L absence de soudure la séquestration ou le rejet du transplant signent les échecs evidents. Mais on peut aussi assister à des échecs relatifs lorsque le transplant se soude à l os porteur et se trouve assimile il convient de tenir compte de l'importance

des détails de cette évolution lesquels doivent être comparés à ceux de l'assimilation des autotransplants frais réalisés dans les mêmes conditions. Il est évident qu'une telle comparaison ne peut être effectuée que sur le plan expérimental (chien). En outre, elle n'a de valeur que pour des transplantations de grandes dimensions, les petits implants osseux étant en pratique toujours réhabités. Ce qui importe en effet n'est pas le démarrage de la réaction ostéogénique du porteur presque toujours constatable, mais bien sa poursuite et son étendue. Les réussites chez le chien et chez l'homme comme les échecs sont extrêmement démonstratifs.

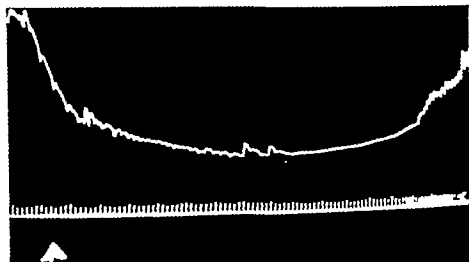


FIG. 1

Chute de la pression artérielle de type anaphylactique observée chez un chien, porteur d'une hétérogreffe aortique constituée par un fragment carotidien de veau, et éprouvée par voie intraveineuse à l'aide d'un extrait antigénique bovin (avec l'aimable autorisation des *Annales de Chirurgie de la Semaine des Hôpitaux*)

c) l'aspect macroscopique du greffon après mort spontanée ou sacrifice du chien porte-greffe. Dans les cas favorables les greffons d'aorte ou de carrefour ne présentent ni dilatation, ni thrombose, à l'inverse des greffons fixés sur des sujets hyperimmunisés. De leur côté les transplants osseux s'incorporent entièrement à l'os porteur et s'adaptent aux exigences mécaniques de leur fonctionnement Dans l'éventualité d'un échec, l'évolution clinique est protéiforme fracture et pseudarthrose lyse pure et simple du transplant, séquestration élimination etc. Cette multitude d'éventualités ne peut être observée dans le cas de greffes artérielles l'hémorragie mettant précocement terme à toute évolution

d) l'histologie du greffon qui illustre dans tous les cas la mort de celui-ci

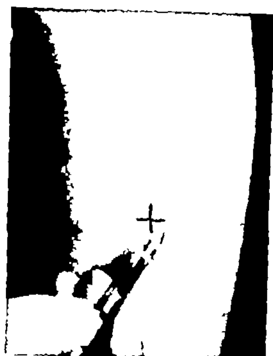


FIG. 2.

Réaction allergique retardée (type tuberculinique) observée chez un homme porteur d'une greffe fémorale à greffon carotidien de veau et sollicité 24 heures au préalable par l'injection intradermique d'un extrait antigénique bovin (avec l'aimable autorisation des *Annales de Chirurgie de la Semaine des Hôpitaux*)

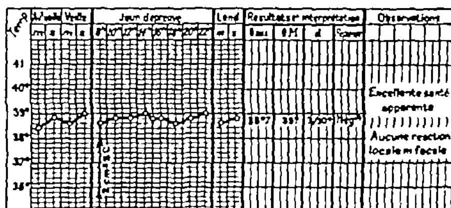


FIG. 3.

Réaction allergique négative chez un chien hétérogreffé Chien n° 12 hétérogreffé sur aorte par carotide de veau lyophilisée depuis 10 mois Encore en vie Éprouvé 6 mois après la greffe avec un extrait aqueux d'artère de veau à pH = 8 (avec l'aimable autorisation des *Annales de Chirurgie de la Semaine des Hôpitaux*)

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B — *Tests sérologiques*

Ils se proposent de mettre en évidence des anticorps anti-espèce dans le sang des greffés. Cette méthode est sujette à erreurs car elle révèle

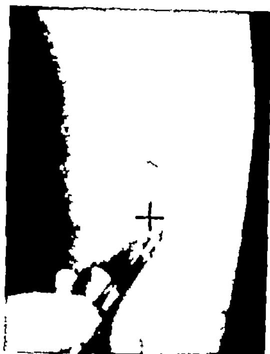


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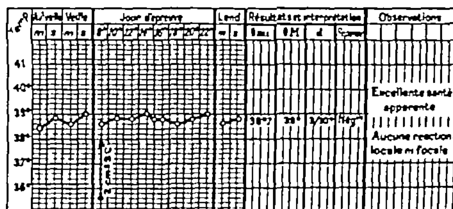


FIG. 3.
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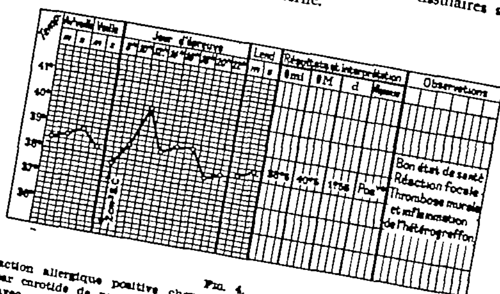


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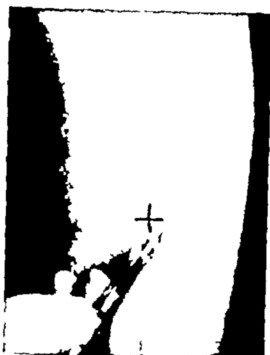


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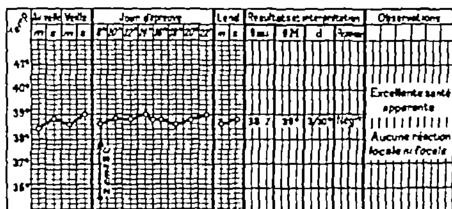


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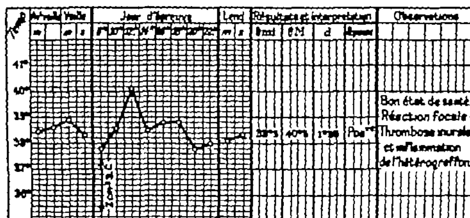


Fig. 4.

Réaction allergique positive chez un chien hétérogreffé. R hétérogreffé sur aorte par carotide de veau lyophilisée depuis 2 mois. Eprouvé le 3e mois après la greffe avec un extrait d'artère de veau à pH = 8 (avec l'aimable autorisation des *Annales de Chirurgie de la Semaine des Hôpitaux*)

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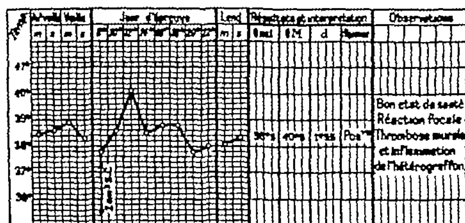


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avec indifférence les anticorps supports authentiques de l'immunité et les anticorps simples témoins épiphénomènes accompagnant la réaction immunologique.

1) *Greffes artérielles*

A l'aide d'un extrait d'antigène d'artère de veau fraîche broyée au mixeur en eau physiologique à pH 7,8 et filtrée 2 types de réactions ont été interrogés

a) La réaction de fixation du complément du type Kolmer semi quantitative à 100 % d'hémolyse

b) La réaction d'hémagglutination passive sur hématies de mouton support visible d'un film antigénique préalablement déposé à leur surface et réagissant *éventuellement avec les anticorps présents dans le* sérum à expertiser

2) *Greffes osseuses*

On a eu recours aux mêmes techniques sérologiques que pour les artères en utilisant diverses préparations antigéniques pulpe splénique broyat artériel moelle osseuse *délipidée*.

C. — *Tests allergiques*

Ce terme générique désigne à son tour deux ordres de tests

a) Test anaphylactique (allergie brutale immédiate) développant dans les cas positifs une réaction « explosive » après injection intraveineuse de l'extrait antigénique d'artère Cette réaction ne fut jamais enregistrée *cependant avec suffisamment de netteté par la simple observa*tion de l'animal Il fallut recourir à l'enregistrement de la pression artérielle qui subit quelquefois dans le cas des greffes artérielles (1) un effondrement spectaculaire à ne pas confondre avec la banale perturbation déclenchée par le choc protéique

b) Test allergique proprement dit (allergie retardée du type tuberculinique) exclusivement utilisé dans le cas des greffes artérielles L'attitude différente de l'homme et du chien devant la réaction tuberculinique nous incita *à adopter une technique propre à chaque espèce*

Chez l'homme une intradermo-réaction locale par injection dans le derme du bras de 1/10 à 2/10 ml de l'extrait antigénique Une réaction positive se signale par une papule blanchâtre avec auréole *inflam*matoire entre 48 et 72 heures.

(1) Des réactions urticariennes ont été notées par Jewer chez l'homme après transplantation d'os de poulain De telles réactions n'ont jamais été observées dans nos transplantations

Chez le chien une réaction totale obtenue après injection hypodermique de 3 à 5 ml d'antigène et groupant des signes généraux (faibles ou nuls) thermiques (nets) locaux (pratiquement absents) et surtout focaux. En effet, au niveau du greffon, le processus inflammatoire est intense et aboutit à la thrombose murale voire à la mort du sujet.

Là encore des tests accessoires de contrôle et de recherche ont permis d'interroger occasionnellement l'aspect du myélogramme, le titre de la properdine, et des quatre portions du complément, le protéinogramme des sérums ainsi que les résultats, fort inégaux, de la corticothérapie et de l'injection, préalablement à la greffe, d'antigène spécifique, dans le but de créer une « paralysie immunologique » dont nous nous expliquons plus loin.

III. — Résultats

1. *Greffes artérielles.*

C'est seulement dans un cas sur 40 chez le chien et dans un cas sur 50 chez l'homme que des réactions immunitaires anti-espèces caractérisées et concordantes ont été relevées chez le porte-greffe. Ces résultats significatifs impliquent la viabilité des hétérogreffes vasculaires en technique chirurgicale humaine, mais incitent également à un contrôle immunologique des greffes car, pour rares que soient ces réactions, elles n'en existent pas moins et constituent une menace quelquefois mortelle. Dans le dessein d'écarter ce danger, nous avons précisé les 2 caractères de ces réactions :

1) *Intensité* révélée par l'hyperpositivité de tous les tests immunologiques, sérologiques ou allergiques. Cette intensité est d'autant plus nette que le greffé présentait avant l'intervention un certain taux d'immunité spécifique, en rapport avec une pathogénie que nous envisageons plus loin.

2) *Précocité,* puisque c'est entre le 1er et le 3e mois que se développent ces manifestations. Aussi avons-nous en mains tous les éléments d'un contrôle immunologique pré- et post-opératoire, basé sur tout ou partie des tests ci-dessus décrits.

a) En effet le contrôle pré-opératoire des futurs greffés se légitime par la reconnaissance, rare (< 1%) de stigmates d'immunité de faible intensité chez des individus neufs n'ayant apparemment eu aucun contact antérieur avec des antigènes d'espèce bovine. Il serait alors aléatoire, peut-être dangereux, de pratiquer une hétérogreffe bovine chez ces sujets déjà sensibilisés et montrant de faibles réactions sérologiques (hémagglutination passive surtout à +) et allergiques (intradermo-réaction faiblement positive). Le mécanisme de cette sensibilisation spécifi-

que spontanée et occulte est ignoré mais une hypothèse peut être avancée pour l'expliquer. L'alimentation carnée (viande de veau ou de bœuf) sous forme de grillades, n'ayant pas dénaturé « à cœur » par la chaleur les protéines spécifiques d'espèce, pourrait être à l'origine, chez les hépato-intestinaux chroniques de *cette immunisation* (2), *ainsi* que certains médicaments d'origine bovine (sérums extraits d'organes).

b) Le contrôle post-opératoire permet de déceler très tôt dès la première semaine une immunité anormalement élevée chez le sujet fraîchement greffé et de suivre la cinétique de ces réactions. Dès lors il sera facile *de se tenir prêt à intervenir devant cet échec immunologique qui* met en péril l'évolution de la greffe.

La recherche immunologique en ce domaine se trouve donc dans une impasse puisqu'elle se bornera à constater et à signaler les échecs effectifs ou probables d'une hétérogreffe vasculaire. Certes elle peut rendre des services signalés mais la réussite chirurgicale trouve le chirurgien satisfait de cette prothèse morte et toutes tentatives de poursuivre une telle étude risquent de le laisser indifférent. L'atténuation ou la disparition des antigènes du greffon sous l'effet de traitements appropriés conservateurs des structures voire de la vitalité des tissus, d'une part et d'autre part le traitement du porte greffe dirigé pour réduire ses réactions spécifiques constituent autant de recherches dogmatiques sans retentissement immédiat sur la chirurgie des hétérogreffes, seulement dignes d'intérêt dogmatique et extrapolables aux greffes (d'organes en particulier). Cette opinion est confirmée par l'avènement des greffes en plastique de synthèse (*crylor* surtout), utilisant des greffons économiques, malléables neutres et sans doute incapables d'induire des réactions d'intolérance (3).

2 *Greffes osseuses.*

Chez le chien les résultats des examens sérologiques anaphylactiques et allergiques sont demeurés constamment *négatifs*. *Les* examens correspondants n'ont jamais été pratiqués chez l'homme devant la réussite régulière des transplantations thérapeutiques d'os réfrigéré ou lyophilisé. Le problème reste entier en ce qui concerne l'explication des échecs cliniques de certaines hétérotransplantations expérimentales pratiquées chez le chien.

(2) On connaît la sensibilisation du cobaye à ces mêmes protéines lorsque cette espèce est nourrie avec de la viande. D'autre part, il est intéressant de rappeler la présence spontanée de sensibilisatrices anti-mouton chez les arabes qui préfèrent cette sorte de viande — fait qui a considérablement gêné le diagnostic sérologique de la syphilis chez ces populations.

(3) *Seuls des solvants très toxiques peuvent solubiliser le crylor* ce qui interdit tout espoir d'obtenir un antigène apte à *révéler* par la sérologie ou l'allergie la réaction de l'organisme à cette prothèse.

Il serait digne d'intérêt d'examiner dans une étude biologique élar gie aux végétaux et aux animaux inférieurs, les similitudes et les dispa rités de chaque type de greffes en fonction de quelques-unes des condi tions qui ont présidé à leur réalisation (traitement du greffon par exem ple). Nous nous bornerons ici à discuter succinctement les caractéristi ques principales des greffes artérielles et osseuses — les plus utilisées en chirurgie — et aussi de donner un aperçu de l'ampleur des problèmes ouverts.

I. — GREFFES ARTÉRIELLES ET OSSEUSES

1 *Similitudes et disparités dans les hétérogreffes artérielles et osseuses*

La triple réaction histologique suturale, endartérielle et adventitielle qui isole le greffon artériel doit le faire considérer comme une *prothèse séquestre tolérée* La constitution de ces lésions, vraisemblablement dues à la réaction immunitaire, peut être aussi considérée comme une sorte de lésion d'arrêt qui s'opposerait à la fuite tardive d'antigènes immu nisants.

En revanche, la réhabitation du greffon artériel est nulle, alors que le greffon osseux est entièrement réhabité par des fusées cellulaires médullifuges, qui s'organiseront plus tard en néo-systèmes haversiens Quels que soient le type, l'origine ou le mode de préparation du trans plant, ce dernier passe toujours par les mêmes étapes Son évolution est dans tous les cas, régie par les mêmes lois qui sont en fait celles de l'ostéogénèse et de l'ossification physiologiques.

L'origine, la structure, la technique de préparation des os transplan tés n'interviennent que pour faciliter ou entraver certaines phases de cette évolution. Ainsi l'hétérogreffe osseuse est une *prothèse guide réha bitable,* sans délimitation nette du greffon et du porte-greffe, lequel résorbe bientôt, en fait, son greffon entièrement remanié

L'explication de l'indigence ou de l'absence des réactions immuni taires décelables dans l'hétérogreffe osseuse par rapport à l'hétérogreffe artérielle tient peut-être à l'importance du conjonctif cellulaire immé diatement mobilisable dans l'artère alors qu'il demeure discret dans l'os. Le tissu osseux doit certainement sa situation immunologique privilé giée à sa structure et à sa charge minérale, ainsi qu'à sa faible teneur protéique, à la place réduite occupée par ses constituants cellulaires enfin à la lenteur de la réhabitation. Au contraire, l'importance des éléments réticulo-endothéliaux dans la moelle osseuse et la nécessité

d'une réhabitation complète des microcavités par le mésenchyme de l'hôte doivent faire attribuer un rôle de premier plan aux phénomènes d'immunité locale tissulaire ou cellulaire. C'est à l'action du froid sur les constituants cellulaires et surtout nucléaires que semble devoir être rapportée l'influence bénéfique de ces agents de préparation sur les hétérotransplants osseux.

2 *Similitudes et disparités dans les auto homo- et hétérogreffes d'artères et d'os*

Seules les autogreffes constituent des réussites biologiques et immunologiques totales alors qu'en revanche leur valeur chirurgicale est controversée pour des motifs qui sortent du cadre de cet exposé mais qui incitent nombre de chirurgiens à s'adresser aux homo- et hétérogreffes.

a) Sur le plan antigénique le choix des homogreffes — surtout si elles ne s'accompagnent pas de la considération des groupes sanguins du donneur — paraît immunologiquement contestable puisque quantitativement (4) le nombre d'espèces d'antigènes mis en jeu (antigène de groupes d'espèces type Forssmann présents chez les individus A et AB antigènes de groupes d'individus majeurs et mineurs et antigènes individuels) peut être alors supérieur à ceux mis en jeu par l'hétérogreffe (antigène d'espèce exclusivement).

b) Sur le plan biologique le traitement subi par le greffon (ébullition traitement par un agent chimique — réfrigération congélation à — 50 C ou cryodessiccation) paraît présenter par rapport au greffon frais, plus d'importance que le type de greffe mis en jeu (hétéro- homo- et même autoplastique)

En effet les greffons congelés ou cryodesséchés sont toujours mieux tolérés (artères) ou plus rapidement réhabitables (os) que les autres diversement traités Tout se passe donc comme si ces traitements avaient la faculté de conserver le tissu tout en adultérant ou supprimant l'antigène ou l'immunigène.

Cette notion est difficile à admettre puisqu'il est connu que la cryodessiccation particulièrement est le meilleur procédé pour conserver intégrales les propriétés antigéniques des bactéries et des virus. Néanmoins les récents travaux de Billingham Brent et Medawar (1956) paraissent assigner la présence de tels antig[illegible] le noyau des cellules ces antigènes étant supprimés par la [illegible] (50) ou la cryodessiccation. Bien plus [illegible] pu voir [illegible] conditions la cryodessiccation dépa[illegible] et être [illegible] lyse pure et sim[illegible]

ple du transplant intégralement détruit et résorbé par le récepteur. On est donc en droit de penser qu'il s'agit en fait d'une réaction bien plus complexe qu'une simple destruction d'antigènes et de considérer que les traitements conservateurs ou modificateurs des transplants osseux ne font que déplacer un équilibre entre des facteurs multiples et complexes.

3. *Perspectives d'avenir*

Les études sur les homo- et hétérogreffes ne pourront progresser sans le secours des investigations immunologiques destinées à préciser l'« intolérance » du porte-greffe au greffon surtout dans le domaine des greffes endocrines et d'organes entiers (rein par exemple). L'effort devra porter :

a) Sur le greffon dans lequel on s'efforcera de dissocier intégrité des tissus et antigènes tissulaires, ces derniers devant être détruits alors que l'intégrité fonctionnelle des tissus devra être strictement conservée. Cette solution physico-chimique paraît problématique.

Le traitement biologique du greffon par un anticorps destiné à « bloquer » les antigènes in situ est théoriquement peu valable.

Quant à la greffe d'un tissu embryonnaire (greffe tréphoplastique non encore immunologiquement différenciée) elle se heurte encore à de sérieux obstacles techniques et fonctionnels.

b) Sur le porte-greffe, en essayant d'amenuiser la réaction de défense immunologique grâce à des traitements biologiques non spécifiques (cortisone, irradiations, etc.) et surtout spécifiques.

Dans ce dernier but, la « charge » des futurs greffés en antigènes spécifiques afin d'obtenir une véritable « paralysie immunologique » est aléatoire chez des sujets déjà malades comme le sont les futurs greffés. L'utilisation soit d'haptènes non provocateurs d'anticorps mais récepteurs de ces mêmes anticorps en formant des complexes « solubles » soit d'anticorps spécifiques paraît plus séduisante. Enfin il convient de signaler une possibilité de « rapprochement immunologique » du greffon et du porte-greffe au stade fœtal du futur donneur de greffon en accord avec les expériences poursuivies depuis 1953 par Hašek. Dans ce but et sans que des résultats puissent être encore avancés, des extraits du futur greffé (broyat de rate, ganglion, hématies, etc.) ont été injectés au fœtus et au veau nouveau-né non encore immunologiquement différencié. Incapable de s'immuniser contre ces antigènes mais apte à se « rapprocher » immunologiquement d'eux, le futur donneur de greffon livrerait donc un tissu ou un organe peut-être moins susceptible de déclencher des réactions d'intolérance chez le porte-greffe spécifiquement « apparenté » sur le plan immunologique.

CONCLUSIONS.

1 *Les greffes artérielles* et *osseuses mortes*, ou plus exactement *les* transplantations prothétiques hétérogènes tendent aujourd'hui à être largement utilisées en chirurgie humaine (greffe veau/homme) résolument orientée vers la réparation et la prothèse.

2 L'intérêt chirurgical pratique de ces hétérotransplantations est prouvé par le nombre des interventions pratiquées chez l'homme (50 greffes fémorales et 150 greffes osseuses) à l'Hôpital E. Herriot à Lyon à l'aide de greffons congelés ou cryodesséchés, sous couvert d'une expérimentation-pilote chez le chien (40 greffes aortiques 60 greffes osseuses).

Cette réussite réhabilite l'hétérotransplantation par rapport à la greffe homogène ou autogène et permet la constitution de banques d'artères et d'os. Elle souligne aussi la différence fondamentale entre l'hétérogreffe artérielle, séquestre toléré et fixé non réhabité, et l'hétérogreffe osseuse, prothèse réhabitable et guide d'ossification.

C'est l'histophysiologie particulière de chaque tissu qui commande *son comportement dans les transplantations. Mais si les mécanismes his*tologiques qui président à ce comportement, sont clairement discernables presque tout reste à découvrir des facteurs biologiques et chimiques qui entravent ou favorisent ce comportement.

3 L'intérêt immunologique ressortit au caractère exceptionnel de l'apparition de réactions immunologiques d'incompatibilité entre porte-greffe et greffon vraisemblablement due au traitement subi par le greffon (congélation ou cryodessiccation) peut-être alors débarrassé des antigènes hétérospécifiques. Exceptionnelles et négligeables dans les hétérotransplantations osseuses elles peuvent néanmoins se manifester au cours des greffes artérielles où elles se montrent précoces et intenses sur les plans de la sérologie (fixation du complément et hémagglutination passive) et de l'allergie (réaction intradermique). Il en découle l'indication d'un contrôle pré et post-opératoire dans les greffes d'artères.

4 L'intérêt dogmatique concerne la jeune science de l'immunogreffologie et porte sur la nécessité d'harmoniser la terminologie (génétique histocytologique et immunologique) les techniques et les résultats des greffes effectuées sur les végétaux les animaux inférieurs les animaux supérieurs et l'homme.

RÉSUMÉ.

Les problèmes biologiques et immunologiques des hétérotransplants artériels et osseux morts sont étudiés sur le plan expérimental (hétérogreffe veau/chien) et sur le plan chirurgical (hétérogreffe veau/homme)

La réussite chirurgicale régulière de ces greffes s accompagne de réactions immunologiques rares (greffes artérielles) ou nulles (greffes osseuses) sur les plan de la sérologie (fixation du complément et hémagglutination passive) et de l aller gie (réaction locale intradermique chez l'homme, réaction totale sous-cutanée chez le chien) Elle permet la constitution de banques de greffons hétérogènes artériels ou osseux.

La différence fondamentale entre les deux types de greffes ressortit à leur évolution histologique l'hétérotransplant artériel est un séquestre toléré et fixé, non réhabité, l'hétérotransplant osseux une prothèse réhabitable guide d ossifi cation Cette étude s'intègre dans le cadre général de l immunogreffologie jeune science en plein essor

SUMMARY

The experimental (veal/dog heterograft) and surgical (veal/human) aspects of the biological and immunological problems which arterial and bone hetero-grafts give rise to are examined.

The regular surgical success of these grafts is accompanied by immunological reactions which are rare (arterial grafts) or absent (bone grafts) from the point of view of serology (complement fixation and passive hemo-agglutination) and from the point of view of allergy (local intradermous reaction in man total sub-cutaneous reaction in dog). An arterial and bone heterogeneous graft bank is thus made possible.

The fundamental difference between the two types of grafts is due to their histological evolution the arterial heterotransplant acts as a fixed tolerated and no reinhabited deposit. The bone heterotransplant is an inhabitable prothesis which acts as a guide to ossification. This study is integrated in the general picture of immunograftology

DISCUSSION

HYATT In view of Dr Knake s observation of yesterday that diff ion of nutrients into a free graft is adequate only for a depth of 30 400 micra it is evident that the relatively gigantic graft used by clin cians will by no means survive *in toto* Therefore the auto- homo- and heterograft initially share a common and serious disadvantage.

We have attempted to define many of the free graft functions when they are surgically buried beneath the skin by calling these functions a capacity of the bio-dynamic unit of the graft This concept is some what chronologically related to graft-host behaviour As a beginning the graft must be biologically acceptable to the operative site (e.g fresh cancellous bone heterografts are rejected occasionally by physical extru sion from the wound) Further the graft should exert a simultaneous mechanical and dynamic function—dynamic in the biological sense that the graft can both evoke and accept without loss of function a cell re

Évolution des implants intraspléniques de thyroïde chez la lapine adulte

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I. — INTRODUCTION.

Golden et Severinghaus [2] ont montré que les sécrétions produites, après castration, par un implant d'ovaire en territoire préportal sont détruites par le foie. Biskind et Mark [1] ont fait la même observation à partir d'œstrogènes en cristaux introduits dans la rate d'animaux castrés. Un ovaire greffé dans la rate, le pancréas ou le mésentère se développe activement à la faveur du défreinage du département correspondant de la préhypophyse et le rôle de cette glande dans l'hypertrophie de l'ovaire implanté est démontré par le fait que cette autogreffe survit simplement sans activité spéciale si elle est faite sur animal hypophysectomisé puis castré (Desaive 1950).

Cette opération, dite de Biskind, a été réalisée non seulement au moyen de l'ovaire, mais encore avec le testicule et diverses autres formations glandulaires, notamment la thyroïde. En ce qui concerne ce dernier organe, Gabe et Arvy ont montré chez la rate thyroïdectomisée qu'après 90 jours les implants intraspléniques de thyroïde témoignent d'une intense activité que traduisent la hauteur de l'épithélium, le caractère petit ou moyen des vésicules, la rareté de la colloïde, l'abondance des vacuoles de Bensley, la fréquence relative des mitoses et la congestion intense du conjonctif périvasculaire; en même temps s'établit une hypothyroïdie générale se marquant notamment par une surcharge glycogénique du foie, un tassement de la zone glomérulaire et une hyperplasie conjonctive de la zone réticulée interne de la surrénale, une hypertrophie de la préhypophyse avec diminution des cellules éosinophiles et présence de cellules basophiles vacuolisées et enfin une lutéinisation marquée des ovaires.

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Ces constatations sont en faveur d'une part, de la destruction par le foie d'au moins une partie des sécrétions thyroïdiennes, et d'autre part d'une action marquée de l'hormone thyréotrope sur les transplants thyroïdiens.

Craps et Martin [8] se sont occupés du même problème et ont confirmé dans leur ensemble les conclusions des auteurs précédents. Ils ont procédé par comparaison de transplants intraspléniques avec ou sans conservation d'un lobe thyroïdien in situ et ont observé dans ce dernier cas au 14e jour de la greffe, une évolution vers le type histologique d'adénome fœtal. Ils ont admis que les transplants sont sécrétants, mais que la sécrétion thyroïdienne n'est pas particulièrement détruite par la glande hépatique.

Nous-même (avec Mewissen et Closon) avons, en 1952 examiné chez la Lapine après strumectomie, le comportement d'un des lobes de la glande introduit dans la rate, au cours des 80 premiers jours de survie de l'animal. Ces premières recherches nous ont permis de constater que l'évolution des auto-transplants intraspléniques de thyroïde se fait en trois phases successives. La première phase dure approximativement 5 jours et correspond à la dégénérescence de la presque totalité de l'implant. La seconde phase, déjà en cours au 10e jour est caractérisée par la coexistence d'un processus central de résorption des parties dégénérées et d'un processus périphérique et centripète de régénération à partir du tissu épithélial sous-capsulaire. La troisième phase débute en général vers le 50e jour et est caractérisée par une tendance à l'achèvement de la réintégration de l'implant dans ses dimensions primitives.

Nous avons en outre observé que l'implant reconstitué est ordinairement de structure alvéolaire, à vésicules petites et moyennes. Et nous avons pu prouver le caractère nettement fonctionnel de l'implant non seulement par le type structurel spécial de son épithélium, mais encore par l'affinité de ce dernier pour le radio-iode^{131}.

Le présent travail apporte à ces premières observations un complément de résultats portant sur une période longue de 940 jours.

II — Matériel et techniques

Soixante-deux lapines, de 2 kilos en moyenne, ont été opérées sous anesthésie locale à la novocaïne à 1 %. Nous avons pratiqué dans la même séance opératoire :

1. la thyroïdectomie totale après ligature des 4 pédicules vasculaires par dissection des deux lobes étroitement accolés chez le lapin aux faces latérales de la trachée et du cartilage thyroïde.
2. l'implantation d'un des lobes (l'autre servant de témoin est immédiatement fixé au liquide de Bouin) dans la rate expo

petite incision médiane épigastrique et ouverte à son pôle supérieur par une minuscule incision sagittale le lobe est *enfoncé à « frottement dur »* dans le parenchyme splénique et l ouverture de la rate est fermée par une ligature au catgut fin

— La thyroïdectomie n est pas toujours bien supportée par le lapin si les récurrents sont lésés, il n est pas rare de voir s établir une pneumonie grave.

— Nous avons, à intervalles variables, prélevé la rate (et son implant) les ovaires, les surrénales et l'hypophyse lorsque l animal montrait des signes de défaillance thyréoprive, de l amaigrissement une pneumonie tardive nous l avons sacrifié sans attendre la mort mais dans la plupart des cas, les prélèvements ont été faits à des dates établies d avance.

— Les différentes pièces ont été fixées au liquide de Bouin ou de Hollande, incluses dans la paraffine et colorées par une méthode trichromique (l Azan par exemple).

— Les rates ont été coupées parallèlement à leur grand axe donc longitudinalement dans 2 cas seulement nous avons pratiqué des coupes transversales, perpendiculaires au grand axe les coupes étudiées correspondent en général aux plus grandes sections obtenues à travers les implants thyroïdiens.

— Dans un certain nombre de cas, nous avons administré à la lapine avant de la sacrifier une dose variable (de 1/2 à 1 millicurie) d Iode radio-actif I^{131} par voie intraveineuse.

— Deux lapines ont été soumises à un traitement continu par un cancérigène puissant, l acétylaminofluorène (AAF), à raison d une dose journalière de 10 milligrammes, en suspension dans l'huile d olive et *administré par voie gastrique par l'intermédiaire* d une fine sonde en gomme

III — ÉVOLUTION DES AUTO-TRANSPLANTS DE THYROÏDE DANS LA RATE DE LAPINES, PRÉALABLEMENT STRUMECTOMISÉES

Au cours de notre étude des auto-transplants intraspléniques de thyroïde chez la lapine adulte thyroïdectomisée nous avons poursuivi quatre buts essentiels

1 Déterminer le comportement général de l'implant et plus spécialement analyser son mode de croissance tout en examinant les aspects *successifs de son architecture du 2 au 940 jour après l opération de* Biskind

2 Évaluer d après les tests histologiques habituels et d après la réponse à l Iode radio-actif I^{131} la valeur fonctionnelle [illegible] lum vésiculaire

3° Rechercher dans quelle mesure le transplant peut, sous l'influence de l'hormone thyréotrope, passer à un état hyperplasique ou même tumoral

4 Analyser l'influence éventuelle de la thyroïdectomie, suivie de l'implantation d un lobe dans la rate, sur l état endocrinien du porteur

Nous allons relater nos résultats à ces quatre points de vue.

A. — *Comportement général de l'implant thyroïdien intrasplénique sa croissance, son architecture*

Par analogie avec la technique que nous avions utilisée anterieurement pour l ovaire (que nous implantions en entier dans la rate) nous avons systématiquement greffé l un des lobes de la glande strumique, avec sa capsule et le tissu cellulo-graisseux y attenant. Nous avons, en effet remarqué que l ovaire greffé en totalité commence par dégénérer et par perdre son appareil folliculaire évolutif seuls persistent l épithélium germinatif sous-péritonéal quelques éléments du hile et les follicules primordiaux du cortex à partir desquels se reconstitue, dès le 18° jour le dispositif folliculaire qui peut conduire à la formation de follicules de de Graaf (en petit nombre d ailleurs) et même — très rarement il est vrai — à la rupture spontanée de ceux-ci. En utilisant un lobe thyroïdien complet nous espérions pouvoir préciser plus clairement qu à partir de petits fragments de glande, les différentes phases de la reconstitution du tissu strumique cet espoir n a pas été déçu

Voici les aspects successifs que nous avons observés au niveau de l'implant thyroïdien intrasplénique du 2 au 940° jour après l opération de Biskind et schématisés sur la figure 1

a) Dans les premières heures qui suivent son implantation à frottement dur dans le tissu splénique le lobe thyroïdien est comme suspendu dans l'hématome qui résulte du traumatisme opératoire. Très rapidement le tissu noble dégénère les vésicules s affaissent, leur épithélium s aplatit et desquame, la structure épithéliale typique de la glande disparaît et est remplacée par une masse amorphe les travées conjonctives s'hyalinisent et la capsule sur laquelle elles s appuyent se désagrège un tissu de granulation et des vaisseaux jeunes apparaissent entre le parenchyme splénique blessé et son contenu thyroïdien quant aux éléments cellulo-graisseux, ils perdent leur contenu lipidique et se présentent comme un treillis en nid d abeille, de localisation et d'importance variables avec les hasards du prélèvement chirurgical de l'implant au niveau de la region cervicale.

Cette phase de dégénérescence glandulaire s'installe immédiatement après l'implantation et est pratiquement achevée entre le 15° et le 18° jour s'il est presque toujours permis avant cette date, de constater la

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présence, en l'un ou l'autre point de la périphérie de l'implant d'un îlot de tissu épithélial il est probable qu'il s'agit là d'un phénomène de rémanence plutôt que d'un processus de régénération débutante

b) A un moment qu'il est difficile de fixer exactement (parce qu'il varie avec les circonstances opératoires et est retardé notamment par une hémorragie intrasplénique importante ou par l'emploi — pour résequer

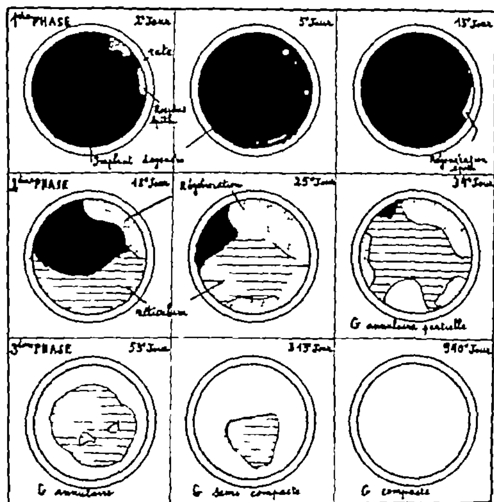


FIG 1

le lobe thyroïdien — du bistouri diathermique qui peut altérer les couches les plus externes de l'épithélium vésiculaire) mais qui semble voisin du 18e jour commence la phase proprement dite de reconstitution du tissu thyroïdien implanté A cette époque la loge splénique d'implantation est une cavité elliptique le plus souvent très allongée dont la paroi contient en l'un ou l'autre point *et très fréquemment au contact même du parenchyme splénique* des vésicules thyroïdiennes de section circulaire ou elliptique à grand axe généralement perpendiculaire à la paroi de la loge

Tout le reste de cette cavité est occupé d une part par l ancien tissu noble dégénéré qu envahissent des éperons vasculaires et des histiocytes et d autre part, par les reliquats capsulaires et cellulo-graisseux qui s organisent en un tissu réticulé, à mailles très fines, pauvres en cellules conjonctives fixes.

Pendant cette période, on assiste simultanément à la multiplication des éléments épithéliaux neufs souvent en mitose et au remplacement des masses dégénérées par le réticulum conjonctif jeune, dont la constitution est généralement complète entre le 35e et le 50 jour

c) Commence alors une troisième phase, dont nous n avions pu en 1952 préciser toutes les caractéristiques, et qui est consacrée au comblement de la loge (résultant de l acte même de l implantation) par le développement du tissu thyroïdien neuf.

Nous avons observé que le mécanisme de cette réhabitation consiste en ordre principal dans une sorte d épibolie des éléments nobles à la surface interne de la paroi de cette loge, au contact même du parenchyme splénique, et en ordre accessoire, dans la colonisation du réticulum conjonctif par des éléments thyroidiens issus des îlots périphériques déjà reconstitués.

Simultanément, des travées conjonctivo-vasculaires, parties du tissu splénique voisin se développent en tous sens au travers de la thyroïde reconstituée et assurent à celle-ci en même temps qu une riche vascularisation une lobulation assez inégale.

En résumé la reconstitution d une glande thyroïde fonctionnelle à partir du lobe implanté dans la rate se fait bien, comme nous l avions pensé en 1952 en trois phases

Au cours de la première phase l implant se nécrose et sa capsule de même que le tissu cellulaire qui l entoure dégénère l ensemble forme au cœur du foyer de contusion que représente dans la rate la cavité traumatique occupée par le lobe thyroïdien greffé un amas amorphe rapidement remanié par les processus de résorption Cette première phase est de courte durée elle couvre en moyenne 15 à 18 jours

Au cours de la deuxième phase trois phénomenes évoluent parallèlement d abord la régénération du parenchyme thyroïdien à partir d'îlots périphériques (vraisemblablement issus des eléments de reserve que toute thyroïde normale détient sous forme de micro-nodules épithéliaux non vésiculaires, le plus souvent au voisinage de la capsule et des grands septa conjonctifs) ensuite la transformation des éléments conjonctifs anciens en un reticulum grele, dont les mailles sont clairement visibles en différents points de l espace d implantation enfin la réhabitation du greffon par des axes conjonctivo-vasculaires partis de l'hôte

splénique Cette deuxième phase commence avec la toute première apparition du tissu thyroïdien vésiculaire neuf s'achève avec la disparition des derniers restes de l'implant dégénéré et s'étend approximativement du 15e au 50e jour après l'opération de Biskind.

Au cours de la troisième phase a lieu la transformation progressive de l'ébauche décrite plus haut en une véritable glande thyroïde intrasplénique pleine et active les éléments épithéliaux progressent par épibolie *sur la paroi interne de la cavité* d'implantation se rejoignent et forment ainsi un anneau périphérique glandulaire autour du tissu réticulé dont les mailles occupent la partie encore libre de cette cavité simultanément d'autres éléments épithéliaux partis des régions périphériques plus précocement reconstituées, colonisent les mailles de ce treillis réticulé Par ce double processus — épibolie et colonisation — la cavité d'implantation se remplit peu à peu de sorte que les coupes longitudinales parallèles au grand axe de l'implant montrent au cours de prélèvements successifs, des aspects partiellement annulaires, puis complètement annulaires et enfin massifs à des stades divers du remplissage

L'apparition d'une thyroïde intégralement achevée et pleine se situe d'après nos observations, aux environs du 500e jour Il faut donc près d'un an et demi pour que, sous l'influence de la thyréostimuline hypophysaire, la cavité d'implantation soit comblée par du tissu thyroïdien la masse de thyroïde ainsi reconstituée est sensiblement voisine de celle que représentaient les deux lobes thyroïdiens primitifs, en place Il ne semble donc pas qu'en ce qui concerne la thyroïde, on soit en mesure par l'opération de Biskind d'obtenir des effets d'hyperdéveloppement *aussi nets que ceux que nous avons constatés avec l'ovaire ce fait tient vraisemblablement à ce qu'une partie seulement des hormones thyroïdiennes* fabriquées par l'implant est détruite dans le foie avec comme conséquence un défreinage incomplet du secteur thyréotrope de la préhypophyse.

Il nous est cependant arrivé de constater que le greffon thyroïdien se développe au point de faire saillie sous le péritoine splénique et d'être visible par transparence *au travers de ce dernier* mais jamais, jusqu'à présent du moins nous n'avons observé ces boursouflures énormes que donnent à la rate les volumineuses formations atrétiques et kystiques de l'ovaire greffé chez la lapine castrée

B — *Structure des éléments nobles de l'implant leur état fonctionnel*

a) Dans les premiers jours qui suivent la thyroïdectomie et l'implantation d'un *lobe thyroïdien dans la rate la structure* vésiculaire typique de ce tissu disparaît Toutefois, dès le tout début de sa reconstitution le

tissu thyroïdien neuf adopte la disposition vésiculaire caractéristique de sa glande d'origine. Les vésicules sont petites ou moyennes : les plus grandes, nous l'avons écrit plus haut, sont allongées dans un sens normal à la paroi de la cavité d'implantation ; l'épithélium est élevé, plus souvent prismatique que cubique ; la colloïde, à ce stade, n'est pas très abondante ; bref, l'aspect du tissu thyroïdien néoformé est celui d'une glande en place, moyennement active. Cet état persiste pendant toute la durée de la deuxième phase de croissance de l'implant, c'est-à-dire jusqu'aux environs du 150e jour. Puis pendant la 3e phase, au fur et à mesure que la cavité d'implantation se comble de tissu thyroïdien de plus en plus massif et dense, le calibre des vésicules s'atténue en même temps qu'il s'uniformise, tandis que la hauteur de l'épithélium s'accroît. Cette lente évolution se poursuit pendant la première année et la première moitié de la seconde, mais à partir du 425e jour approximativement et à coup sûr à partir du 500e jour, en même temps que deviennent systématiquement pleines ou presque les greffes examinées, la thyroïde est strictement micro-vésiculaire et en hyperplasie manifeste.

b) L'aspect histologique du transplant thyroïdien intra-splénique est donc celui d'une glande fonctionnellement active ; nous étudions en ce moment une technique nous permettant de doser les hormones thyroïdiennes dans le sang veineux d'origine splénique, dans le foie et à la sortie du foie ; en attendant les résultats de cette expérience, la preuve *formelle* de la valeur physiologique du greffon ne peut être fournie que par l'appréciation directe de la fixation par l'implant de l'iode exogène administré à l'animal sous forme d'iode radioactif.

On sait que la thyroïde fixe la plus grande partie (80 à 90 %) de l'iode corporel, fait important dont il résulte que l'I^{131} jouit d'une affinité toute particulière pour cette glande, qu'elle soit en place ou ectopique. Les rayonnements gamma et bêta émis par l'isotope de l'iode sont capables d'impressionner une émulsion photographique et cette propriété permet d'apprécier, au moins qualitativement, la présence du métalloïde métabolisé par le tissu thyroïdien.

D'une manière générale, une réponse positive à l'I^{131} est liée à la présence de colloïde (Fitzgerald et Foote [4]) et, par voie de conséquence, à l'architecture vésiculaire et alvéolaire de la glande ; toutefois cette condition n'est pas indispensable en ce sens qu'une glande à disposition trabéculaire peut (exceptionnellement il est vrai) fixer l'iode I^{131} (Fitzgerald et Foote [4]) et que par ailleurs, des vésicules colloïdes apparemment typiques se montrent quelquefois réfractaires à cette réaction (Dargent, Berger, Moret et Guinet [3]).

Il semble donc qu'une autohistoradiographie thyroïdienne positive après introduction d'iode radioactif ne fasse que traduire le passage de

l'épithélium par un état fonctionnel, dont la vésicule colloïde est le plus souvent mais pas obligatoirement la manifestation morphologique.

Nous avons sur plusieurs de nos lapines opérées suivant la technique de Biskind utilisé la technique histo-autoradiographique de Leblond, Findlay et Gross[11] qui consiste à vernir au moyen d'une émulsion photographique une coupe histologique de thyroïde après traitement de l'animal par l'I^{131}. Nous avons 48 heures avant le prélèvement de la rate, administré à la lapine un millicurie d'I^{131} en intraveineuse. La rate enlevée a été fixée au Bouin puis débitée suivant les procédés ordinaires. Les coupes colorées ont été enduites d'émulsion photographique, gardées en chambre noire pendant 96 heures puis traitées par un révélateur et enfin montées au baume de Canada.

Nous donnons ici trois résultats de cette technique respectivement après 53, 77 et 192 jours.

Au 53e jour l'implant (T 15) offre une structure microvésiculaire et la distribution des grains d'argent réduit correspond sensiblement à celle des vésicules colloïdes.

Au 77e jour l'implant (M 26) présente un aspect trabéculaire pauvre en vésicules et cependant la fixation de l'isotope s'y montre extrêmement intense.

Au 192e jour l'implant (M 21) montre une disposition vésiculaire polymorphe et la répartition des grains d'argent ne correspond pas nécessairement aux vésicules les plus riches en colloïde ; au contraire de nombreux foyers de concentration de l'isotope apparaissent à des endroits pratiquement avésiculaires.

D'après ces observations il se confirme donc que les implants thyroïdiens intraspléniques après thyroïdectomie sont très actifs, que cette activité (au moins pour ce qui concerne l'iode extrinsèque) apparaît précocement, et que son siège est l'épithélium lui-même en ce sens que la concentration de l'iode radio-actif n'est nullement conditionnée par le volume des vésicules et l'importance de leur contenu en colloïde.

C. — *Hyperplasie et caractères précancéreux du tissu thyroïdien implanté.*

a) De temps à autre et même dans les implants les plus compacts on découvre l'une ou l'autre vésicule très large, de type franchement kystique et de signification incertaine bien que la présence de colloïde accumulé dans sa lumière ne permette pas de mettre en doute son origine thyroïdienne.

Dans les greffons les plus anciens l'aspect général est celui de l'*adénome fœtal* et la présence (au 940e jour) d'un épaississement marqué de la paroi d'un grand kyste semble indiquer une amorce de végétation

TABLEAU I — *Caractéristiques essentielles des implants (G) de lobe thyroïdien dans la rate, après thyroïdectomie chez la lapine adulte*

Numéros d'ordre	*Durée d'observation (en jours)*	*Architecture générale de la greffe (G)*	*Diamètre moyen des vésicules (en μ)*	*Hauteur moyenne de l'épithélium (en μ)*	*Divers.*
T 9	4	Dégénérescence complète, un îlot glandulaire persistant.	62,5	8,4	Foyers hémorragiques.
M 12	13	Dégénérescence complète, quelques vésicules disparates.	36,2	6,3	Infiltrats lymphoï des Scléroses hyaline
M 11	18	Dégénérescence complète.	—	—	
T 8	21	Début reconstitution, un gros îlot périphérique en pleine croissance.	33,6	15,3	
M 31	34	Résorption en cours. G annulaire partielle.	45,2	16,4	
T 2	38	Résorption achevée, réticulum constitué G annulaire fragmentée	47 4	18,2 (mitoses)	
T 1	41	G. presque compacte.	49,8	16,6	
M 2	81	G. presque compacte.	57,6	12,45	
M 52	119	G annulaire fragmentaire.	36 6	11 4	
M 53	135	G presque compacte.	56,5	12,15	
M 46 (+ AAF)	161	G presque compacte.	59 1	9,3	
M 20	175	G. annulaire incomplète.	49,8	10,95	Infiltrats lymphoïdes
M 21	192	G annulaire incomplète.	65,7	7 03	
M 32	309	G annulaire fragmentée.	61,3	10,2	Kystes.
M 30 (+ AAF)	313	G à moitié compacte.	58,8	7,29	
M 41	367	G presque compacte.	45,2	9,3	
M 35	402	G annulaire fragmentée	35,2	7,3	
M 48	427	G. presque compacte.	45,6	9 19	
M 44	493	G presque compacte.	36,2	14,4	
M 62	705	G presque compacte.	36,6	14,1	
M 23	940	G compacte	53,6	15,9	

papillaire. Toutefois jusqu'à présent (c'est-à-dire après un peu moins de trois ans d'observation) nous n'avons pu constater la formation de structures de type franchement tumoral.

b) Il est remarquable à ce dernier point de vue de signaler que les deux lapines (M 46 et M 39) que nous avons soumises à un traitement intensif par un cancérigène puissant de la thyroïde l'acétyl-aminofluorène (AAF) (et qui ont reçu régulièrement 160 à 320 mg de ce produit à raison de 10 mg à intervalles de 3 à 6 jours) n'ont pas plus que les autres lapines expérimentées montré de manifestations néoplasiques au niveau de leurs implants, examinés après 151 et 313 jours.

Nous retrouvons ici une observation intéressante de Hall et Bielschowsky [1] qui ont constaté que l'AAF ne produit pas la cancérisation thyroïdienne, mais la favorise lorsqu'une autre technique (la fragmentation de la glande par exemple) a préalablement sollicité l'effet hyperplasiant de la thyréostimuline. *Il est donc probable que si l'AAF n'a pas* aidé à la cancérisation de nos implants, c'est parce que ceux-ci n'avaient pas encore atteint, sous l'influence de l'hormone thyréotrope l'état désirable d'hyperplasie.

c) On accorde actuellement en clinique humaine, une importance particulière aux infiltrats lymphoïdes observables dans la thyroïde soit au cours de la transformation adénomateuse soit même pendant la cancérisation on connaît par ailleurs l'intérêt des éléments lymphomateux dans la constitution des syndromes de Hashimoto, de Mikulicz et de Sjögren. Il n'est donc pas inutile de signaler ici que dans 2 cas (M 40, 178 jours et M 23, 940 jours) nous avons constaté l'existence d'infiltrats lymphoïdes au voisinage de régions épithéliales particulièrement actives.

• • •

(avec basophilie) une hyperplasie intense de la cortico-surrénale et une mise au repos des thyroïdes. Le même type d'intervention, pratiqué au moyen de la thyroïde, provoque-t-il des altérations comparables ?

Nous avons régulièrement prélevé, chez nos animaux sacrifiés les ovaires, les surrénales et la préhypophyse au niveau de ces différents organes nous avons fait les observations suivantes

Les ovaires sont systématiquement au repos leur aspect est celui que nous avons toujours observé après thyroïdectomie, chez la lapine. De poids réduit (120 milligrammes en moyenne) l'organe montre une réduction importante de la phase non autonome, d'obédience extra-ovarienne, de son développement folliculaire phase qui commence au stade que nous avons appelé E (et qui correspond à la fin de la période de croissance de l'ovocyte) et s'achève au stade que nous avons appelé G (et qui est le follicule de de Graaf préovulatoire) Les coupes en série que nous avons faites de ces ovaires montrent un nombre réduit de follicules mûrissants les plus grands dépassant rarement le stade E.

Il semble donc que la fraction d'hormones thyroïdiennes qui franchit la barrière hépatique après opération de Biskind (pour la thyroïde) est quantitativement insuffisante ou qualitativement inadéquate pour influencer le mécanisme d'évolution folliculaire.

Les surrénales par contre sont généralement hypertrophiées leur poids moyen dépasse — par unité — 350 mg contre un poids moyen de 225 mg chez les témoins cette hypertrophie porte surtout sur les couches glomérulée et fasciculée externe, comme après opération de Biskind pour l'ovaire. Tout se passe comme si la castration et la thyroïdectomie suivies d'implantation du tissu correspondant en territoire préportal favorisaient, de la part de la préhypophyse, une hypersécrétion d'hormone corticotrope.

La préhypophyse, enfin est extrêmement intéressante son poids augmente et atteint 30 mg en moyenne contre 18 à 20 mg chez les témoins mais sa structure est nettement altérée, particulièrement à partir de la fin de la première année d'observation Les transformations portent avant tout sur la répartition des cellules chromophiles les acidophiles diminuent de nombre et se dispersent, tandis que les basophiles se multiplient.

Nous n'avons toutefois pas constaté la présence d'adénomes hypophysaires chromophobes ou basophiles, comme il en a été observé après thyroïdectomie simple chez le rat

Ces observations encore fragmentaires nécessitent d'importantes additions. Telles quelles elles traduisent un bouleversement endocrinien dont il reste à préciser les modalités, mais qui semble se ramener au niveau de la préhypophyse, à un freinage gonadotrope, à une activité

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* * *

Dans le but de résumer ces différentes notions nous avons rassemblé sur le Tableau I les caractéristiques essentielles d'un certain nombre d'implants classés par ordre chronologique du 4 au 940e jour

Le tableau comprend dans sa partie descriptive 4 colonnes verticales, réservées respectivement à l'architecture de l'implant à la structure des vésicules à la hauteur de l'épithélium glandulaire et à la présence d'éventuelles formations anormales

D — *Influence de la thyroïdectomie suivie de l'implantation d'un lobe dans la rate sur l'état endocrinien du porteur*

Chez la lapine l'opération de Biskind pour l'ovaire entraîne chez le porteur après quelques *mois* d'observation un état particulier caractérisé essentiellement selon nous par une hypertrophie préhypophysaire

(avec basophilie) une hyperplasie intense de la cortico-surrénale et une mise au repos des thyroïdes. Le même type d'intervention, pratiqué au moyen de la thyroïde provoque-t-il des altérations comparables ?

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Ces observations encore fragmentaires nécessitent d'importantes additions. Telles quelles elles traduisent un bouleversement endocrinien dont il reste à préciser les modalités, mais qui semble se ramener au niveau de la préhypophyse à un freinage gonadotrope, à une activité

accrue du secteur corticotrope et à un très net hyperfonctionnement thyréotrope.

IV — CONCLUSIONS.

L'implantation intégrale après thyroïdectomie d'un des deux lobes thyroïdiens dans la rate appartient à ce type d'intervention imaginé par Biskind qui fait intervenir le filtre hépatique pour assurer un défreinage partiel ou complet du secteur préhypophysaire correspondant à la glande implantée et pour réaliser sous l'influence des sécrétions libérées de ce département pituitaire l'hyperplasie de l'implant. Nous avons étudié les variations de structure du lobe greffé et le comportement de l'animal expérimenté au cours d'une période longue de 940 jours et nous avons pu faire à ce propos les observations suivantes

1° Après une courte phase de dégénérescence presque intégrale le tissu thyroïdien régénère à partir d'îlots de tissu épithélial proches de la capsule. Ces îlots s'appuyent sur le parenchyme splénique lui même et se développent par épibolie sur la paroi de la cavité d'implantation tandis que s'achèvent la résorption des zones dégénérées et l'organisation en un fin réticulum des restes conjonctifs et cellulo-graisseux de l'implant cette seconde phase commence vers le 5° jour et se termine aux environs du 50° jour Au cours d'une troisième phase le comblement de la cavité d'implantation se réalise par un double mécanisme la constitution par glissement progressif d'une enveloppe périphérique de tissu noble et la colonisation par ce même tissu des mailles du réticulum conjonctif qui parcourent la loge d'implantation.

2° La structure du tissu noble est d'abord à la fois micro- et macro-vésiculaire et est comparable à celle d'une glande thyroïde en place moyennement active. Mais dès que commence la troisième phase elle tend à s'uniformiser et à devenir de plus en plus micro-vésiculaire. Il faut toutefois attendre les approches du 500° jour pour que cet aspect devienne constant

Parallèlement l'épithélium tend à devenir uniformément prismatique et à montrer une activité constructive de plus en plus grande

3° Sur le plan fonctionnel la technique à l'iode radioactif I^{131} a prouvé que l'activité du tissu thyroïdien reconstitué est considérable mais qu'elle n'est pas — quant à la concentration de l'iode exogène — exclusivement liée à la présence de vésicules et à la richesse de celles-ci en colloïde

4° A l'exception de rares images de végétations papillaires débutantes dans quelques kystes atypiques, nous n'avons pas constaté —

jusqu'à la limite de 940 jours — de transformation tumorale des implants, même après préparation de l'animal par l'acétyl aminofluorène.

5 Dès la fin de la première année d'observation, les lapines expérimentées montrent en général un syndrome endocrinien caractérisé par une hypotrophie ovarienne, une hyperplasie modérée de la cortico-surrénale et une hyperplasie préhypophysaire combinée à une diminution des cellules acidophiles et à une augmentation des cellules basophiles sans constitution d'adénomes chromophobes.

6 Ces constatations plaident en faveur d'une modification considérable par le filtre hépatique des sécrétions hormonales issues de l'implant thyroïdien intrasplénique des recherches sont en cours pour tenter de préciser l'importance quantitative et la portée qualitative de ces altérations.

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DISCUSSION

KROHN Dr Desaive has shown us the evolution of the thyroid graft in the spleen Can he say whether it differs in any respect from the behaviour of a subcutaneous graft in the first few weeks ?

The failure of thyroid grafts to develop tumours in an experimental situation known to favour the formation of ovarian tumours suggests that the destruction of thyroid hormone by the liver is incomplete.

accrue du secteur corticotrope et à un très net *hyperfonctionnement* thyréotrope.

IV — Conclusions

L'implantation intégrale après thyroïdectomie d'un des deux lobes *thyroïdiens dans la rate appartient* à ce type d'intervention imaginé par Biskind qui fait intervenir le filtre hépatique pour assurer un défreinage partiel ou complet du secteur préhypophysaire correspondant à la glande implantée et pour réaliser sous l'influence des sécrétions libérées de ce département pituitaire, l'hyperplasie de l'implant. Nous avons étudié les variations de structure du lobe greffé et le comportement de l'animal expérimenté au cours d'une période longue de 940 jours et nous avons pu faire à ce propos les observations suivantes

1 *Après une courte phase de dégénérescence presque intégrale* le tissu thyroïdien régénère à partir d'îlots de tissu épithélial proches de la capsule. Ces îlots s'appuyent sur le parenchyme splénique lui-même et *se développent par épibolie sur la paroi de la cavité d'implantation* tandis que s'achèvent la résorption des zones dégénérées et l'organisation en un fin réticulum des restes conjonctifs et cellulo-graisseux de l'implant cette seconde phase commence vers le 5ᵉ jour et se termine aux environs du 50 jour Au cours d'une troisième phase le comblement de la cavité d'implantation se réalise par un double mécanisme la constitution par glissement progressif d'une enveloppe périphérique de tissu noble et la colonisation par ce même tissu des mailles du réticulum conjonctif qui parcourent la loge d'implantation.

2 La structure du tissu noble est d'abord à la fois micro- et macro-vésiculaire et est comparable à celle d'une glande thyroïde en place *moyennement active Mais dès que commence la troisième phase*, elle tend à s'uniformiser et à devenir de plus en plus micro-vésiculaire il faut toutefois attendre les approches du 500ᵉ jour pour que cet aspect devienne constant

Parallèlement l'épithélium tend à devenir uniformément prismatique et à montrer une activité constructive de plus en plus grande

3 Sur le plan fonctionnel la technique à l'iode radioactif I^{131} a prouvé que l'activité du tissu thyroïdien reconstitué est considérable *mais qu'elle n'est pas* — quant à la concentration de l'iode exogène — exclusivement liée à la présence de vésicules et à la richesse de celles-ci en colloïde

4 A l'exception de rares images de végétations *papillaires débutantes* dans quelques kystes atypiques nous n'avons pas constaté —

jusqu à la limite de 940 jours — de transformation tumorale des implants, même après préparation de l animal par l acétyl aminofluorène.

5 Dès la fin de la première année d observation les lapines expérimentées montrent en général un syndrome endocrinien caractérisé par une hypotrophie ovarienne, une hyperplasie modérée de la cortico-surrénale et une hyperplasie préhypophysaire combinée à une diminution des cellules acidophiles et à une augmentation des cellules basophiles sans constitution d adénomes chromophobes

6 Ces constatations plaident en faveur d une modification considérable par le filtre hépatique, des sécrétions hormonales issues de l implant thyroïdien intrasplénique des recherches sont en cours pour tenter de préciser l importance quantitative et la portée qualitative de ces altérations.

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DISCUSSION

KROHN Dr Desaive has shown us the evolution of the thyroid graft in the spleen. Can he say whether it differs in any respect from the behaviour of a subcutaneous graft in the first few weeks ?

The failure of thyroid grafts to develop tumours in an experimental situation known to favour the formation of ovarian tumours suggests that the destruction of thyroid hormone by the liver is incomplete

and there the cortical layer is pierced by openings which enable the nutrient vessels to enter. In the centre haematopoietic cells reappear they are separated from the bone by an almost continuous layer of osteoblasts.

In this way a typical ossicle is formed

By observing the development of this autologous graft for several months (the longest period of observation being eight months), only minor changes were noticed until the osseous tissue had become constituted of lamellae of the secondary type. The shape of the ossicle persisted without changing and this was also true of its substance and of that of the central haemopoietic marrow. At no time did this ossicle formed from an autologous graft, set up any inflammation in its vicinity. Ossification was 100 per cent successful (fifty times in fifty rats)

When experiments are made in other sites such as muscle the subcutaneous area the spleen kidney mesentery and epicranium, the autologous graft of bone marrow develops in an identical manner

After remaining for several months in these different environments *it gives no sign of necrobiosis nor of minor deterioration suggesting* approaching absorption. We have every reason to regard this ossicle as *permanent*

In other kinds of animal the rabbit and the guinea-pig the autologous graft of bone *marrow develops in an identical manner and* always culminates (ten times out of ten) in the formation of a similar ossicle.

These facts observed in bone marrow proved equally true of the periosteum[4] After the trabecular ossification stage and osteoclastic rearrangement a true ossicle is formed in which a dense and continuous cortical layer surrounds a central medullary cavity. After three months there are no signs of absorption nor inflammation suggesting approaching degeneration

Finally fracture fibrocartilage[5] behaves in the same way and also results in the formation *of an ossicle* with no signs of deterioration even after seven months observation

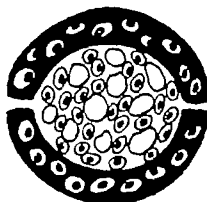
So with three completely different kinds of tissue (bone marrow periosteum and fracture fibro-cartilage) we observe an ossification of the grafted tissue and the growth of a tissue complex possessing the complete structure of an ossicle.

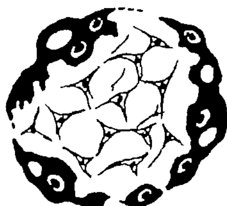
This phenomenon is invariable and seems to have an unlimited life-span

Quite different however is the histological development of a transplant taken from another animal of the same species.

The homologous graft

The first difference from the autologous series of grafts is found in the inconstancy of the ossification process. Here 15 per cent of homologous marrow grafts in the rat came to nothing through absorption quite independently of any technical reasons for failure It is agreed that this is due to an immunity reaction set up in the host animal





From examination of the ossified grafts, we have discovered they have an abnormal aspect which frankly differs from what has been observed in the autologous series. Under the binocular microscope we see that the bone which has developed in the substance of the marrow has been obliged to assume the latter's shape and presents rounded contours. But the vascular openings are large and irregular instead of being small and round Under the microscope, the structure of an ossicle is revealed an osseous cortical layer surrounding a central cavity Development has therefore proceeded up to this stage.

But two histological differences immediately appear to the eye of the observer First the bony shell is extensively eroded by osteolysis. The vitality of the bone is seriously impaired and some osteoplasts have lost their bone cell Sometimes necrobiosis is complete.

Secondly the central cavity is no longer filled with active haemopoietic marrow This has given way *to fibrous tissue normally* poor in cells In certain cases, the graft has become inflamed and penetrated by *leucocytes, even to the centre* Sometimes however this invasion is very slight.

Whatever the intensity of the reaction in the host the development unfailingly ends in necrobiosis and total absorption of the graft

This phenomenon is invariable

Before continuing let me add for the sake of completeness that no heterologous graft (a transplant between animals of different species) has ever produced bone *finally that devitalization by heat intense cold* (—190 C) and alcohol irremediably destroys osteogenetic powers.

It is universally agreed that the destruction of a homologous graft is the result of an immunity reaction set up in the host animal This principle rests upon experimental and theoretical bases which are so well established that it would be superfluous to describe them here[1] This is the explanation of the resistance of the animal organism to the heterologous graft and its behaviour towards the homologous graft In our experiments, this reaction against the grafted tissue is particularly apparent The bone indeed *appears* as a very characteristic tissue unique of its kind. It is particularly well defined and cannot be confused with neighbouring tissues *consequently its deterioration is very easily* observed Because of its hardness its absorption will take a long time and the successive stages are spread over a long period it thus becomes possible to follow its course over several weeks even several months. The new formed bone in a homologous graft shows a slow histological development one can easily grasp the ways in which it differs from an autologous graft.

From the theoretical point of view it is possible to deduce from this that the new formed bone is distinguishable from the host by a *biological individuality inherited from its homologous origin* This proves that the homologous bone derives from the grafted tissue

This comparison leads to a reflexion *if the new bone was being* formed from cellular elements surrounding the graft by metaplasia of young mesenchymatous cells it would be autologous since it arises from the host's own tissues It should then behave identically whether the inducting graft was autologous or homologous but this, however is contradicted by the facts

This shows the weakness of the theory of metaplasia by induction

Conclusion.

A graft of osteogenetic tissue (bone marrow periosteum or fracture fibro-cartilage) leads to the formation of a complete ossicle. In autologous transplants, the osseous tissue and central haemopoietic marrow are permanent. In homologous transplants the new formed bone and marrow are doomed to be absorbed This phenomenon is particularly clear with bony tissue in which the slow pace of absorption permits lengthy observation.

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DISCUSSION

Medawar I should like to call attention to the importance of Dr Danis's work from an embryological point of view The so-called « induction » of bone, though it occurs in the adult, has repeatedly been cited as a process equivalent to embryonic induction. Dr Danis's work shows that some examples of « bone induction » represent no more than the survival of grafted tissue they are not of inductive origin But does this apply to all such examples ? I should like Dr Danis to comment upon the formation of heterotopic bone in the rabbit in response to gall bladder epithelium and possibly also to alcoholic tissue extracts. A second question is anything known about the *type* of cell in marrow which is responsible for osteogenesis ?

Danis I know that many experiments have been done on rabbits in which it seems to be true that new bone forms around *dead* transplants—but I have never observed this phenomenon after study of many species. As to induction by alcoholic extracts—alcohol *alone* has been said to work, so the effect cannot be specific.

In answer to the second question—this problem is now under investigation in my laboratory

HYATT The accomplishment of Dr Danis demonstrates the absence of clinical usefulness in the living homograft. It may live long enough to form bone but like *most viable homografts ultimately succumbs* with destruction of the bone graft.

In view of the observations of Mosiman Urist and Ray we have developed some reservations on their experimental site because of the following

(a) Initially the environment is for all practical purposes tissue culture *in vivo* and initially enjoys some exemption from destructive immunological abilities.

(b) When vascularization occurs, and occurrence is inevitable the *homograft response as we have come to know it and as you demonstrated* is accomplished

From the standpoint of induction I wonder whether the new bone we see is the result of a predestined role of a cell type or types or does the graft evoke a response from a previously non-orientated (bone) primitive cell type.

DANIS I know McClean's experiments giving some evidence that immature mesenchymal cells may become osteogenetic but this has not been my experience.

ALBERT I should like also to congratulate Dr Danis on his clear demonstration that bone is surely formed by the donor marrow cells introduced in the anterior chamber of the eye of an animal of another strain *If that bone had been formed by an induction of the host cells* there would be no reason why that bone had not the same evolution as an autograft The delayed resorption of that bone is characteristic of an homograft and that merits our attention

This raises indeed the whole problem of the mechanism of the relative tolerance to homografts introduced in the anterior chamber of the eye, the role of the vascularization in the early or delayed break down of homografts and also the problem of adaptation raised earlier by Dr Woodruff

Even in the privileged position of the anterior chamber of the eye where homologous marrow cells not only survive but give rise to the formation of real homologous bone the latter will finally be destroyed by a real delayed transplantation reaction This shows that neither the special nutritional conditions of the anterior chamber of the eye nor a good secondary vascularization can avoid the breakdown of the homograft. And that delayed resorption is rather disappointing from Dr Woodruff's point of view considering that after a prolonged survival homografts should be able to undergo a kind of adaptation That delay

ed destruction of newly formed homologous bone in the anterior chamber of the eye is rather similar to the delayed breakdown of certain skin homografts between two different strains with rather few or weak genetical differences

WOODRUFF I do not doubt that the bone formed in Danis's experiments was of donor origin but it would be wrong to conclude that there is no such thing as heterotopic ossification. Perhaps the best known example is ossification occurring in the vicinity of an autograft of bladder epithelium, first reported as far as I remember by Huggins. We have seen ossification sometimes in the necrotic medulla of an adrenal graft, and very occasionally in the anterior chamber of the guinea pig eye after destruction of a thyroid homograft.

DANIS I have never seen bone formation in or around liver fascial muscle or lymph node grafts—that is around « soft tissues ». But the ease with which marrow produces bone suggests that the cases cited by Dr Woodruff might be due to the action of circulating marrow cells which have penetrated into an inflamed area. Marrow cells do in fact appear in blood smears from stressed rabbits

ALBERT The suggestion of Dr Danis of a kind of bone marrow embolism with secondary bone formation in an inflammatory or traumatic region can be admitted in some very special conditions and can then also be compared to the fact that a damaged tissue will experimentally fix the major part of an intravenous injection of living microbes. But I am not quite sure that this may give the whole explanation of all heterotopic bone formations, even if there is no reason of a mobilisation of bone marrow cells

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